BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/54, 15/82, A01H 5/00 — A1

(11) International Publication Number: WO 96/21023

(43) International Publication Date: 11 July 1996 (11.07.96)

(21) International Application Number: PCT/NL96/00012

(22) International Filing Date: 8 January 1996 (08.01.96)

(30) Priority Data:
95200015.6 6 January 1995 (06.01.95) EP
(34) Countries for which the regional or international application was filed: NL et al.
95200762.3 27 March 1995 (27.03.95) EP
(34) Countries for which the regional or international application was filed: NL et al.

(71) Applicant *(for all designated States except US)*: CENTRUM VOOR PLANTENVEREDELINGS- EN REPRODUKTIEONDERZOEK (CPRO - DLO) [NL/NL]; Postbus 16, NL-6700 AA Wageningen (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: VAN TUNEN, Arjen, Johannes [NL/NL]; Droevendaalsesteeg 1, NL-6708 PB Wageningen (NL). VAN DER MEER, Ingrid, Maria [NL/NL]; Droevendaalsesteeg 1, NL-6708 PB Wageningen (NL). KOOPS, Andries, Jurriaan [NL/NL]; Droevendaalsesteeg 1, NL-6708 PB Wageningen (NL).

(74) Agent: DE HOOP, Eric; Octrooibureau Vriesendorp & Gaade, P.O. Box 266, NL-2501 AW The Hague (NL).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AZ, BY, KZ, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DNA SEQUENCES ENCODING CARBOHYDRATE POLYMER SYNTHESIZING ENZYMES AND METHOD FOR PRODUCING TRANSGENIC PLANTS

(57) Abstract

A DNA fragment is disclosed having a nucleotide sequence SEQ ID No. 1 as shown in Figure 4A or a homologous sequence having a similarity of at least 70 % encoding 1-sucrose:sucrose fructosyltransferase. Also disclosed is a DNA fragment having a nucleotide sequence SEQ ID No. 2 as shown in Figure 4B or a homologous sequence having a similarity of at least 70 % encoding 1-fructan:fructan fructosyltransferase. Further this invention discloses a recombinant DNA comprising one or more of said DNA fragments, or comprising said DNA fragment(s) in the inverted orientation. Using said fragments transformed organisms can be produced showing a modified fructan profile.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

Title: DNA sequences encoding carbohydrate polymer synthesizing enzymes and method for producing transgenic plants.

This invention relates to nucleotide sequences encoding fructan synthesizing enzymes, a recombinant DNA sequence comprising one or more of said nucleotide sequences, a method for producing a genetically transformed host organism showing a modified fructan profile, and transformed plants or plant parts showing said modified fructan profile.

Fructans refer to a group of carbohydrate compounds in which one or more fructosyl-fructose linkages constitute a majority of the linkages. Fructans are fructosepolymers with usually, but not necessarily, one terminal glucosylunit [G-$(F)_n$, G = optional, n≥2]. The fructosyl-fructose linkages in fructans are of the ß-2,6 or ß-2,1 linkage type. Fructans with predominantly ß-2,6 fructosyl-fructose linkages are usually called levan(s). Fructans with predominantly ß-2,1 fructosyl-fructose linkages are usually called inulin(s).

Fructan biosynthesis is common in several bacterial, fungal and algal families and also in specific plant families, such as the Liliaceae (e.g. *Allium cepa*), Poaceae (e.g. *Lolium perenne*) and Asteraceae (e.g. *Helianthus tuberosus*). The function of fructans in bacteria and fungi is poorly understood. It has been suggested that fructans act as extracellular stores of carbohydrate that can be mobilized

during periods of carbohydrate stress (Jacques, 1993). In plants, fructans can function as reserve carbohydrates which serve as a source of carbon for (re)growth (Meier and Reid, 1982). In fructan-storing crops, fructan synthesis is restricted not only to specific organs (e.g. the stems or tubers of *H. tuberosus*, the bulbs of *Allium spp*, the leaf bases and stems of grasses) but also specific cell types within these organs (usually the parenchyma cells). In these specific cell types, the vacuole is probably the location of both fructan biosynthesis and storage (Darwen and John, 1989; Wagner et al., 1983).

In bacteria, examples of fructan synthesizing bacteria are *Streptococcus mutans* and *Bacillus subtilus*, the biosynthesis of fructans from sucrose is catalysed by only one enzyme: levansucrase (EC 2.4.1.10) in *B. subtilus* (Dedonder,1966) and levansucrase, but also called fructosyltransferase (FTF, EC 2.4.1.10) in *S. mutans* (Carlsson, 1970). Bacterial fructan synthesis proceeds via the direct transfer of fructose from a donor-sucrose (G-F) to sucrose or other acceptor molecules according to the following reversible reaction:

$$n(G\text{-}F) + \text{acceptor} \rightarrow n(G) + \text{acceptor-}(F)_n, \quad (1)$$

where n may be larger than 10.000.

Water, hexoses, sucrose, oligosaccharides and levan may act as acceptor molecules for fructosyl units from sucrose (fructosyl donor).

Bacterial DNA sequences encoding FTF in *S. mutans* and levansucrase in *B. subtilus* are already described in the literature (Sato and Kuramitsu, 1986; Steinmetz et al. 1985). Bacterial genes from several sources were used to transform specific host plants which normally cannot synthesize fructans, thereby inducing fructan synthesis (see for example: Van der Meer et al., 1994; Ebskamp et al., 1994). A method to enhance the solid content of tomato fructs, using the levansucrase gene from *B. subtilus* and the dextransucrase gene from *Leuconostoc mesenteroides* is described in application WO

89/12386. A method to modify the fructan pattern in plants which normally cannot synthesize fructans, using the levansucrase-encoding ftf gene from *S. mutans* and the levansucrase-encoding *SacB* gene from *B. subtilus* is described in applications NL A 9300646 and WO 94/14970. The use of a levansucrase-encoding DNA sequence from *Erwinia amylovora*, which after integration in the host plant genome leads to the synthesis of levans, is described in DE 4227061 A1 and WO A 9404692. In all said applications, transgenic plants are described which are transformed with levansucrase genes from bacteria. Accordingly, these transgenic plants synthesize and accumulate fructans structurally comparable to those synthesized by the donor bacteria (Van der Meer et al., 1994; Ebskamp et al., 1994).

The present application differs from said applications in that it is related to fructosyltransferase-encoding DNA sequences derived from plants. These enzymes are structurally different from bacterial enzymes since there is no significant homology at the amino acid level and DNA level. Besides, the mechanism of fructan biosynthesis in plants is essentially different from that in bacteria. In contrast to fructan biosynthesis in bacteria, the formation of fructans in plants is mediated by more than one enzyme. For example, in *Helianthus tuberosus* (the Jerusalem Artichoke), fructan biosynthesis is catalysed by two enzymes: sucrose:sucrose fructosyltransferase (SST, EC 2.4.1.99) and fructan:fructan fructosyltransferase (FFT, EC 2.4.1.100). The SST and FFT from *H. tuberosus* are involved in the synthesis of ß-2,1 linked fructans (inulin) and are therefore also designated as 1-SST and 1-FFT. 1-FFT has been purified from tubers of *H. tuberosus* (Lüscher et al., 1993, Koops and Jonker, 1994). The purification of SST has proven more difficult to achieve. A putative SST has been purified, at a very low yield, from several plant sources (Shiomi and Izawa, 1980; Praznik et al., 1990; Angenent et al., 1993). However, in none of these studies the purity of the enzyme has convincingly been shown. Furthermore, it has not conclusively been shown in these

studies that the isolated enzyme does not represent an invertase.

Large quantities of 1-SST and 1-FFT have now been purified up to homogeneity from tubers of *H. tuberosus* (1-FFT: Koops and Jonker 1994) and their reaction mechanisms extensively investigated. 1-SST from *H. tuberosus* catalyses the initial step of fructan biosynthesis, the synthesis of the trisaccharide 1-kestose (1-[G-(F)$_2$]) from two molecules of sucrose (G-F), according to the following reaction:

$$G\text{-}F + G\text{-}F \rightarrow 1\text{-}[G\text{-}(F)_2] + G, \quad (2)$$

wherein G-F = sucrose, -F = fructosylunit, -G = glucosylunit, G = glucose

1-SST can also catalyse the formation of the tetrasaccharide 1,1-[G-(F)$_3$] and pentasaccharide 1,1,1-[G-(F)$_4$] (Fig. 3A). Therefore, 1-SST activity can be described by the following general reaction:

$$G\text{-}(F)_n + G\text{-}(F)_m \rightarrow G\text{-}(F)_{n-1} + G\text{-}(F)_{m+1}, \quad 1 \leq n \leq 3, \; 1 \leq m \leq 3 \quad (3)$$

It has also been found that 1-SST from *H. tuberosus* to some extent can catalyse the transfer from a fructosyl unit from G-(F)$_n$, $1 \leq n \leq 3$, onto water.

The second enzyme, 1-FFT, catalyses the formation of fructans with a higher degree of polymerization. This enzyme catalyses a polymerization reaction by the transfer of fructosyl units between trisaccharides, tetrasaccharides and larger fructose polymers according to the following general reaction:

$$G\text{-}(F)_n + G\text{-}(F)_m \rightarrow G\text{-}(F)_{n-1} + G\text{-}(F)_{m+1}, \; n \geq 2, \; m \geq 2 \quad (4)$$

It has also been found that 1-FFT catalyses the transfer of fructosyl units between sucrose (G-F) and galactose (Gal)-containing carbohydrates [(Gal)$_n$-G-F], also called galactans. For example, 1-FFT can catalyse the transfer of a fructosyl

unit from G-$(F)_2$ onto raffinose (Gal-G-F) which results in the formation of [Gal-G-$(F)_2$]. It cannot be excluded that both 1-SST and 1-FFT from *H. tuberosus* can use other substrates as fructosyl acceptor.

Although 1-SST and 1-FFT have some overlapping activity - both enzymes can catalyse the formation of tetra and pentasaccharides (reactions 3 or 4) - 1-SST and 1-FFT are distinctly different enzymes. The 1-SST and 1-FFT proteins have different physical properties and are encoded by different genes. 1-SST and 1-FFT have essentially different enzymic properties. 1-FFT is not able to catalyse the initial step of fructan synthesis (reaction 2), whereas 1-SST is not able to catalyse the formation of fructan polymers with a degree of polymerization higher then 5 [G-$(F)_n$, $n>4$]. In conclusion, with 1-SST activity alone, it is only possible to synthesize oligofructans from sucrose with a degree of polymerization of up to 5 [G-$(F)_n$, $2 \leq n \leq 4$]. To synthesize fructans with a higher degree of polymerization and using sucrose as a substrate, both 1-SST and 1-FFT are needed. With 1-FFT activity alone, it is not possible to synthesize fructans from sucrose. It was found by the present inventors that protein fractions containing purified 1-SST as well as purified 1-FFT could use sucrose as a sole substrate for the synthesis of fructans with a degree of polymeriazation of at least 15 [G-$(F)_{14}$, Fig. 3B].

Bacterial fructans differ from fructans in plants with respect to the degree of polymerization and branching type and, consequently, in chemical and physical properties. In general, fructans from plants are assembled from less than 1000 fructosylunits. Fructans from *H. tuberosus* are assembled from less than 100 fructosylunits. Fructans synthesized by bacteria may comprise more than 10.000 fructosyl units. Plant and bacterial fructans therefore differ in their possible applications. For fructans with a relatively low degree of polymerization, such as those isolated from Asteraceae (e.g. Jerusalem Artichoke, chicory or dahlia), an application as phosphate substitute in calcium binding agents and detergents

has already been worked out (WO91/17189). Other applications are related to the organoleptical properties of fructans. The sweetening strength of fructans G-$(F)_n$ decreases with an increasing degree of polymerization (increasing n-value). The sweetening strength of the oligofructans G-$(F)_2$ and G-$(F)_3$ approximates that of sucrose (G-F). The very long chain fructans such as those occurring in bacteria are not sweet at all. Very short chain fructans, such as those synthesized by sucrose:sucrose fructosyltransferase can therefore be used as sweeteners with the additional advantage that these sweet-tasting fructans are non-cariogenic and can withstand digestion in the digestive tract of humans, which opens possibilities for use as a low caloric sweetener. The short chain fructans, and also the longer chain fructans, can be used as the hydrophilic moiety of biosurfactants.

In contrast to the bacterial genes encoding levansucrase, which have already been cloned, the genes encoding SST and FFT have not been isolated before from plants. We found that the SST and FFT-encoding genes from plants, at the amino acid level, have no significant similarity to the known levansucrases and, at the DNA level, have no significant degree of homology to the levansucrase genes. For this reason it has not been possible to isolate the fructosyl transferase genes from plants using heterologous levansucrase probes from bacteria. It has also not been possible to isolate SST and FFT-encoding genes from plants using the amino acid sequences of the purified SST and FFT enzymes and their deduced oligonucleotide primers. The reason for this is that, although methods have been described for the purification of fructosyl transferases from plants, it has not been possible so far to obtain SST and FFT enzymes in sufficiently large amounts and with sufficiently high degrees of purity.

The object of this invention is to provide nucleotide sequences encoding SST and FFT.

Another object of this invention is to provide nucleotide sequences, obtained by recombination or mutagenesis of nucleotide sequences encoding SST or FFT, which encode enzymes

having fructosyltransferase activity.

Yet another object of this invention is to provide a method for transforming non-fructan synthesizing crops into fructan-synthesizing crops by introduction of the SST and/or FFT-encoding genes.

Yet another object of this invention is to (partly) switch off the fructan synthesis in crops which normally synthesize fructans.

Accordingly, this invention provides a DNA fragment having a nucleotide sequence SEQ ID. No. 1, as shown in Fig. 4A, or a homologous sequence having a degree of homology of at least 70% which encodes 1-sucrose:sucrose fructosyltransferase. Further, this invention provides a DNA fragment having a nucleotide sequence SEQ ID. No. 2 as shown in Fig. 4B or a homologous sequence having a degree of homology of at least 70% which encodes 1-fructan:fructan fructosyltransferase.

1-Sucrose:sucrose fructosyltransferase (1-SST) and 1-fructan:fructan fructosyltransferase (1-FFT) were purified from tubers of *Helianthus tuberosus*. Purified enzymes were cleaved into peptides by tryptic digestion, and the resulting peptide mixtures were separated by HPLC. N-terminal amino acid sequencing was performed for selected peptides. Amino acid sequences specific for 1-SST and 1-FFT were used to design degenerated oligonucleotide primers specific for 1-SST and 1-FFT, respectively, for use in RT-PCR. PCR was performed using cDNA as template, a tail-specific primer and the degenerated primers specific for either 1-SST or 1-FFT. First-strand cDNA was synthesized from poly(A)$^+$RNA isolated from tubers from *H. tuberosus*. With the 1-SST-specific primer, RT-PCR resulted in a 450 bp specific fragment. With the 1-FFT-specific primer, RT-PCR resulted in a 800 bp specific fragment. The 450 and 800 bp PCR fragments were subsequently used to screen a cDNA library made from *H. tuberosus* tubers to isolate the full length cDNA sequences encoding 1-SST and 1-FFT, respectively.

The SST and FFT-encoding sequences from plants of the present invention induce, after insertion into the genome of a host organism, for example a plant, changes in the

concentrations of carbohydrates containing at least one fructosyl unit (sucrose, oligofructans, fructans or galactans), or cause a change in the degree of polymerization of oligofructans, fructans or galactans. The present invention is related to said carbohydrates, since sucrose is the substrate for SST, oligofructans are products of SST, oligofructans and fructans with a higher degree of polymerization are substrates and products of FFT. Besides, said fructosyltransferase enzymes can also perform transfructosylation reactions with galactans of the raffinose series as fructosylacceptor.

The present invention includes DNA sequences which are at least 70% identical to the 1-SST-encoding sequence from *H. tuberosus*, irrespective of whether the homologous sequences are derived from other plant sources, or obtained by mutagenesis of fructosyltransferase-encoding sequences from plant sources or from microorganisms. It is preferred that the degree of homology is at least 80%, more preferred that the degree of homology is at least 85% and still more preferred that the degree of homology is at least 90%. It is most particularly preferred that the degree of homology is at least 95%.

The present invention includes DNA sequences which are at least 70% identical to the 1-FFT-encoding sequence from *H. tuberosus*, irrespective of whether the homologous sequences are derived from other plant sources, or obtained by mutagenesis of fructosyltransferase-encoding sequences from micro-organisms. It is preferred that the degree of homology is at least 80%, more preferred that the degree of homology is at least 85% and still more preferred that the degree of homology is at least 90%. It is most particularly preferred that the degree of homology is at least 95%.

The present invention includes also DNA sequences obtained by *in vivo* and *in vitro* recombination using SST and/or FFT-encoding sequences from plants and fructosyltransferase-encoding sequences from other prokaryotic or eukaryotic sources, including bacteria and fungi.

The present invention relates to SST-encoding DNA sequences from plants, which after insertion into the genome of a host organism induce the synthesis of oligofructans comprising of 2, 3 and/or 4 fructosyl units [G-(F)$_n$, 2≤n≤4]. The present invention also relates to FFT-encoding DNA sequences from plants which after insertion into the genome of a host organism together with SST-encoding DNA sequences, induce the synthesis of fructans with a higher degree of polymerization [G-(F)$_n$, n>4]

The present invention also relates to chimeric gene constructs comprising sequences encoding SST or FFT, or part of the sequences, the sequences being present in the antisense orientation. Introduction of these antisense constructs in plants which can synthesize fructans, will cause inhibition of SST or FFT catalysed reactions or cause inhibition of SST or FFT expression.

The present invention relates to chimeric gene constructs encoding SST, or part of the sequence, the coding sequence being present in antisense orientation. Introduction of these antisense constructs into the genome of host plants which can synthesize fructans, such as species of the Asteraceae, Liliaceae and Poaceae family, reduce or block the conversion of sucrose into oligofructans [G-(F)$_n$, 2≤n≤4]. Since only SST is able to catalyse the first step of fructan synthesis (reaction 2), in such transgenic plants, also the synthesis of fructans with a higher degree of polymerization, [G-(F)$_n$, n>4], will be reduced or blocked and these plants will accumulate sucrose rather than fructans.

The present invention relates to chimeric gene constructs encoding FFT, or part of the sequence, the coding sequence being present in the antisense orientation. Introduction of these antisense constructs into the genome of host plants which can synthesize fructans, reduce or block the conversion of oligofructans [G-(F)$_n$, 2≤n≤4] into fructans with a higher degree of polymerization [G-(F)$_n$, n>4]. Transgenic plants thus obtained will accumulate oligofructans, rather than fructans with a higher degree of polymerization.

Accordingly, the present invention provides a method for producing a genetically transformed host organism showing a modified fructan profile, which comprises the steps of:

i) preparing a chimeric gene construct comprising one or more DNA fragments as defined above, or said DNA fragments in the inverted orientation, operably linked to a promotor sequence active in said host organism and a terminator sequence active in said host organism,

ii) introducing the chimeric gene construct into the genome of the host organism.

The host organism may be a microorganism or a plant. In case the host organism is a plant, the method additionally comprises the step of

iii) regenerating the transformed plant cells to transgenic plants.

More specifically the method of the invention comprises the following steps:

a. construction of a chimeric gene comprising essentially the following sequences:
   a promoter which ensures the formation of a functional RNA or protein in the intended target organism, target organs, tissues or cells,
   a DNA sequence encoding SST or FFT,
   a transcription terminator operationally connected to the DNA sequence, the SST or FFT-encoding DNA sequence being functionally connected to a promoter,
   a DNA sequence encoding a targeting signal or a transit peptide which ensures targeting of SST or FFT to a specific subcellular compartment;

b. introduction of the chimeric gene into the genome of a host organism so as to obtain genetic material comprising the DNA sequence and

c. regeneration of the genetic material in a transformed host organism.

In the recombinant DNA of the present invention, the DNA sequence encoding SST or FFT is preferably linked to a

regulatory sequence that ensures proper expression of the DNA sequence in a host organism, such as a bacterium, a yeast, an alga or a plant, at a sufficiently high expression level. Regulatory sequences are a promoter, a termination signal and a transcription or translational enhancer. A promoter can be the 35S promoter of the cauliflower mosaic virus (CaMV), or a sugar-inducible promoter like the patatine promoter or an organ-specific promoter like the tuber-specific potato proteinase inhibitor II promoter or any other inducible or tissue-specific promoter.

In the recombinant DNA of the present invention, the SST or FFT-encoding DNA sequence is preferably linked to regulatory sequences that are operative in plants and that ensure proper expression of the DNA sequence in the different plant organs, tissues or cells. A highly preferable promoter is a promoter which is active in organs and cell types which normally accumulate sucrose (the primary substrate for fructan synthesis). The production of fructans is particularly advantageous in organs storing large amounts of sucrose, such as the tap roots of sugar beet or the stems of sugar cane. Besides roots, other organs or cell types are involved in the synthesis, processing, transport and accumulation of sucrose. Therefore, SST or FFT-encoding sequences are also suitably expressed in leaves, stems, roots, tubers, reproductive organs, and seeds.

In the recombinant DNA of the present invention, the DNA sequence encoding SST or FFT contains, or is linked to, a sequence encoding a transit peptide which directs the SST or FFT mature protein to a subcellular compartment containing sucrose. The production of fructans is particularly advantageous in the vacuole which can accumulate very high concentrations of sucrose (up to 900 mol m^{-3}). Besides the vacuole, other subcellular compartments are involved in the synthesis (cytoplasm), processing (cytoplasm, mitochondria, plastids) and transport (cell wall, cytoplasm) of sucrose. The present invention therefore relates to the use of sequences that allow targeting of the SST or FFT product to specific

subcellular compartments, such as the vacuole, the cell wall, mitochondria, plastids and cytoplasm.

The present invention also relates to gene constructs, comprising a sequence encoding SST or FFT, or part of the sequences, the coding sequence being present in the antisense orientation. In these gene constructs, the SST or the FFT-encoding sequence is preferably linked to a promoter that ensures the formation of an antisense RNA in the cell types which normally were able to synthesize fructans.

The recombinant DNAs of the present invention may also encode proteins having herbicide resistance, plant growth promoting, plant growth inhibiting, anti-fungal, anti bacterial, anti-viral and/or anti-nematode properties or conferring stress resistance. The recombinant DNAs of the present invention may further encode proteins which induce sterility. In case that the DNA is to be introduced into a heterologous organism it may be modified to remove known mRNA instability motifs (such as AT rich regions); and polyadenylation signals, and/or codons which are preferred by the organism, into which the recombinant DNA is to be inserted, are used so that expression of the thus modified DNA in the host organism is higher than that obtained by expression of the unmodified recombinant DNA in the same host organism.

The present invention also provides the transformed host organism, especially a transformed plant, produced by the above method. The invention preferably includes agricultural, forage, vegetable, ornamental and fruit crops, more preferably sugar beet, sugar cane, potato, petunia, alfalfa, soybean, rice, ryegrass, thimothygrass, wheat, barley, sorghum, maize, chicory, Jerusalem artichoke, tulip, melon, onion, garlic, tomato, strawberry, apple and pear. Moreover, the invention includes a plant cell, seed, fruit, seedling or any plant part harbouring recombinant DNA, comprising a sequence encoding SST or FFT as defined herein. Further, this invention includes the progeny of the transformed plants which contain the DNA stably incorporated and heritable in a Mendelian manner, and/or the

seeds of such plants and such progeny.

EXPERIMENTAL

Purification of 1-SST

Tubers of *Helianthus tuberosus* 'Colombia' were used for the extraction of 1-SST and 1-FFT. Tubers were harvested in August-September, during the period of rapid tuber growth and massive fructan accumulation. Tubers were washed and frozen in liquid nitrogen. Four hundred gram of frozen tubers (-80°C) were pulverized and immediately thereafter homogenized in a Waring blender in 900 cm^3 50 mol m^{-3} phosphate (P) buffer, pH 6.5, containing 10% (w/v) glycerol, 1 mol m^{-3} $MgSO_4$, 1 mol m^{-3} Na_2EDTA, 1 mol m^{-3} PMSF (Sigma, USA) 1 mol m^{-3} DTT, 1.5% (w/v) PVPP and 20 mol m^{-3} $Na_2S_2O_5$. The homogenate was filtered through three layers of Miracloth and centrifuged at 17,000 g for 1 h.

The protein extract was kept at 4°C and adjusted to 45% saturation with $(NH_4)_2SO_4$. The insoluble proteins were pelleted by centrifugation (10,000 g, 30 min) and discarded. The 45% supernatant was brought to 70% saturation by further addition of $(NH_4)_2SO_4$. The pellet, obtained after a second centrifugation step, was redissolved in 60 cm^3 50 mol m^{-3} phosphate (P) buffer, pH 6.5, 1 mol m^{-3} DTT and 1 mol m^{-3} PMSF (Sigma, USA), and desalted by dialysis against 10 mol m^{-3} P-buffer, pH 6.5, 1 mol m^{-3} PMSF and 1 mol m^{-3} DTT, for 16 h. After buffer replacement, dialysis was continued for another 3 h. The whole procedure was performed at temperatures between 0 and 4°C. The centrifuged dialysate (30,000 g, 30 min) was applied onto a 25x120 mm Q Sepharose Phast Flow column (4°C), which had been prewashed with 10 mol m-3 bis Tris, pH 6.5, 1 mol m^{-3} DTT, 1 mol m^{-3} PMSF and 5 mol m^{-3} EDTA in Milli Q water. Bound proteins were eluted with an NaCl gradient (0-300 mol m^{-3}) in the same buffer at a flow rate of 5 cm^3 min^{-1}. 1-SST eluted at 200-250 mol m^{-3} NaCl.

The Q Sepharose fractions were adjusted to 400 mol m^{-3} with solid $(NH_4)_2SO_4$. Fractions of 20 cm^3 were loaded onto a 15 x 50 mm column of Phenyl Sepharose High Performance or Phenyl

Sepharose High Substitution, which where pre-equilibrated with 10 mol m^{-3} bis Tris buffer, pH 6.5, containing 500 mol m^{-3} $(NH_4)_2SO_4$, 1 mol m^{-3} DTT, 1 mol m^{-3} PMSF, 2 mol m^{-3} EDTA and 0.1% CHAPS (buffer A) at 12°C. Elution of bound proteins was carried out using a linear gradient of buffer A (100-0%) without $(NH_4)_2SO_4$, containing 25% (v/v) ethylglycol, at a flow rate of 1 cm^3 min^{-1}. 1-SST eluted at 100 mol m^{-3} $(NH_4)_2SO_4$.

Phenyl Sepharose fractions up to 10 cm^3 were injected onto a 5 x 50 mm Concanavalin A Sepharose column, prewashed in 20 mol m^{-3} bis-Tris, pH 6.5, 250 mol m^{-3} NaCl, 0.5 mol m^{-3} $CaCl_2$, 0.5 mol m^{-3} $MnCl_2$, 1 mol m^{-3} DTT and 1 mol m^{-3} PMSF. Bound 1-SST was eluted with 500 mol m^{-3} α-CH_3-mannopyranoside in the same buffer.

Active fractions of one Concanavalin A-run were pooled and applied to a 5x200 column packed with spherical (15 μm) hydroxylapatite (Merck, Germany). The column was pre-equilibrated in 2 mol m^{-3} $CaCl_2$, 10 mol m^{-3} NaCl, 1 mol m^{-3} DTT, 1 mol m^{-3} PMSF and 0.1% CHAPS (buffer A). Proteins bound to the column were eluted with stepped gradient of buffer A and 500 mol m^{-3} potassium phosphate buffer, pH 6.5, at a flowrate of 0.5 ml min^{-1}. 1-SST eluted at 75-100 mol m^{-3} potassium phosphate.

Active fractions of one hydroxylapatite run were pooled and applied onto a 5x50 mm Mono Q-column which was pre-equilibrated with 10 mol m^{-3} P-buffer, pH 6.5, 1 mol m^{-3} DTT, 1 mol m^{-3} EDTA and 0.1% CHAPS. Bound proteins were eluted with an NaCl gradient (0-500 mol m^{-3}) at a flow rate of 0.5 cm^3 min^{-1}. 1-SST eluted at 250 mol m^{-3} NaCl.

All columns, column packings and chromatography equipment were obtained from Pharmacia (Sweden), unless indicated otherwise.

Purification of 1-FFT

A crude protein extract was obtained from tubers of *H. tuberosus* as described for the purification of 1-SST. The supernatant of the crude protein extract, as obtained after centrifugation was adjusted to 45% saturation with $(NH_4)_2SO_4$ and stirred for 1 h. The insoluble proteins were pelleted by

centrifugation at 10,000 g for 30 min. The pellet was redissolved in 60 cm^3 50 mol m^{-3} P-buffer, pH 6.5, 1 mol m^{-3} DTT and 1 mol m^{-3} PMSF, and dialyzed overnight against 10 mol m^{-3} citrate/phosphate (C/P) buffer, pH 4.5, containing 1 mol m^{-3} PMSF and 1 mol m^{-3} DTT. The contents of the dialysis tubing was readjusted to pH 6.5 with 0.2 M Na_2HPO_4 and the insoluble proteins were removed by centrifugation at 30.000 g for 1 h. The supernatant was loaded onto a 25 x 120 mm column of Q Sepharose Phast Flow (Pharmacia) pre-equilibrated with 10 mol m^{-3} P-buffer, pH 6.5, 1 mol m^{-3} DTT and 1 mol m^{-3} PMSF in Milli Q water ((Millipore B.V., The Netherlands). The column was cooled to 4°C. Bound proteins were eluted with a positive NaCl gradient at a flow rate of 5 cm^3 min^{-1}. 1-FFT eluted at 200-250 mol m^{-3} NaCl.

Solid $(NH_4)_2SO_4$ was added to the Q Sepharose fractions to give a final concentration of 750 mol m^{-3} with. Fractions of 5 cm^3 were loaded onto a 15 x 50 mm column of Phenyl Sepharose High Performance (Pharmacia), pre-equilibrated with 10 mol m^{-3} P-buffer, pH 6.5, containing 750 mol m^{-3} $(NH_4)_2SO_4$ and 1 mol m^{-3} DTT. Bound proteins were eluted with a negative $(NH_4)_2SO_4$ gradient at a flow rate of 1 cm^3 min^{-1} at 12°C. 1-FFT eluted at 450-400 mol m^{-3} $(NH_4)_2SO_4$.

Phenyl Sepharose fractions (up to 4 cm^3) were injected onto a Hiload 16 x 600 mm Superdex 75 prep grade column (Pharmacia) pre-washed in 10 mol m^{-3} P-buffer, pH 6.5, and 1 mol m^{-3} DTT. Proteins were eluted in the same buffer at a flow rate of 0.5 cm^3 min^{-1} at 20°C. 1-FFT eluted 95-105 min after injection.

1-FFT and 1-SST assays

The 1-FFT activity of column fractions was routinely assayed at 35°C. Aliquots of 25 mm^3 were mixed with 25 mm^3 0.3 g Neosugar P (Meiji Seika Kaisha, Ltd, Tokyo, Japan; Neosugar consists of 1% hexoses, 4% sucrose, 42% G-(F)$_2$, 44% G-(F)$_3$ and 7% G-(F)$_4$) per cm^3 of 100 mol m^{-3} C/P-buffer, pH 6.5. After 3 h, the reaction was stopped by boiling the incubation mixture in a waterbath for 5 min. A net gain of GF_4 was taken as a measure for 1-FFT activity.

For 1-SST activity, 15 mm^3 column fractions were mixed with 15 mm^3 500 mol m^{-3} GF in 100 mol m^{-3} C/P-buffer, pH 5.0 and incubated for 3 h at 35°C. GF_2 synthesis was taken as a measure for 1-SST activity.

Analysis of sugars and fructans

Sucrose and oligofructans were analyzed by RP-HPLC using a 2.1 x 220 mm Speri-5 RP 18 column (Brownlee Labs, Santa Clara, USA). Milli Q water was used as the eluant at a flow rate of 0.3 cm^3 min^{-1} at 37°C. Glucose and fructose were quantified on a 6.5 x 300 mm Shodex SC-1011 column (Millipore B.V., Waters Chromatography Division, The Netherlands) run at 85°C with Milli Q water at 0.75 cm^3 min^{-1}. Sugars were detected with a 2142 refraction index detector (RID, Pharmacia). Identification of oligofructans was by comparison of their retention times with those from the oligofructans purified from Neosugar P or from *H. tuberosus* and by the glucose/fructose ratios of the individual oligofructans (Koops and Jonker, 1994).

HPAEC analyses of oligofructans and fructans with a higher degree of polymerization were performed on a Dionex Series 4000 ion chromatograph equipped with 250 x 4 mm CarboPac PA1 anion exchange column and a 25 x 3 mm CarboPac PA guard column. Fructans were separated with a 60 min linear gradient of 0.25 to 0.4 mol m^{-3} NaAc in 0.1 mol m^{-3} NaOH at a flow rate of 1 ml min^{-1}. Detection was by pulsed amperometry (PAD) with a gold-working electrode. The applied potential of a pulse was kept at 0.1, 0.6 and -0.6 V for 0.5, 0.1 and 0.05 seconds, respectively. Rhamnose was used as an internal standard. Fructans were identified by comparison of their retention times with those of fructan standards isolated and purified from *H. tuberosus* according to the method of Heinze and Praznik (1991).

Amino acid sequencing of 1-SST and 1-FFT

Mono Q fractions of 1-SST or Superdex 75 fractions of 1-FFT were desalted and concentrated by centrifugation in Centricon-

10 (2 cm^3) ultrafiltration devices (Grace B.V., Amicon division, The Netherlands) and subsequently collected by precipitation in 80% (v/v) aqueous acetone at -20°C. 1-SST and 1-FFT (each 50 µg) were dissolved in SDS-buffer (Laemmli, 1970) and separated on a pre-cast ExcelGel SDS, gradient 8-18. Proteins were stained with Coomassie Brilliant Blue according to Rosenfeld et al. (1992). The stained 1-SST (2 bands; due to its intrinsic lability 1-SST is cleaved by the SDS treatment) and 1-FFT bands were excised with a scalpel and washed, dried and partially rehydrated according to the procedure of Rosenfeld et al. (1992). Sequencing-grade trypsin (0.5 µg; Boehringer, Germany) was added to the gel slice, and in-gel digestion of the proteins was carried out for 4 h at 30°C. The resulting peptides were recovered by two extractions of 20 min each, with 50 µl acetonitrile, water, trifluoracetic acid and Tween 20 (60:40:0.001:0.0002, v/v). The resulting peptide mixture was separated by preparative RP-HPLC on a 9.3 x 250 mm SuperPac Pep-S column (Pharmacia) eluted with a linear gradient of 0.1% TFA in 0 to 60% aqueous acetonitrile at a flow rate of 4 ml min^{-1}. Individual peptide fractions were collected manually and stored at -80°C. The amino acid sequences of selected peptides were determined by Edman degradation, using a model 477A pulse-liquid sequenator connected on-line to a model 120A RP-HPLC unit (Applied Biosystems). Amino acid sequences specific for 1-SST or FTT were translated into the corresponding degenerated DNA sequences (Example 1, Table 1), which, in turn, were used as primers for PCR.

DNA methodology

DNA and RNA isolation, subcloning, restriction analysis and sequencing were performed using standard methods described in molecular biology manuals (Sambrook et al. 1989, Ausubel et al. 1994).

cDNA synthesis

Poly(A)$^+$RNA was isolated from tubers of *H. tuberosus* 'Colombia'. Single stranded cDNA was synthesized by reverse transcriptase from 10 μg poly(A)$^+$RNA by priming the poly(A)$^+$RNA with the following tail specific primer: 5'-CCGAATTCAATACGACTCACTATAGCG$(T)_{15}$-3'

PCR

Degenerated oligonucleotides specific for 1-SST or 1-FFT and the tail specific primer, 5'-CCGAATTCAATACGACTCACTATAGCG-3' were used for amplification of the single strand cDNA. PCR was performed in 50 μl PCR buffer (Life Technologies), containing 100 pmol cDNA template, 100 pmol of the tail specific primer and 100 pmol of primers specific for 1-SST or 1-FFT. Amplification involved 30 cycles of denaturing (1 min, 92°C), annealing (1 min, 42°C) and amplification (1 min, 72°C). The resulting fragments were electrophoresed in 0.7% agarose, excised from the gel, isolated from the agarose matrix and subcloned into pMOSBlue vector (Amersham). 1-SST and 1-FFT specific fragments generated by PCR were used to screen a Uni-ZAP XR cDNA library.

Construction and screening of a cDNA library

Ten μg of poly(A)$^+$RNA isolated from tubers of *H. tuberosus* 'Colombia' was used as starting material for the construction of an Uni-ZAP XR cDNA library (Stratagene). Construction, plating and screening of the library were performed according to the protocols developed by Stratagene (La Jolla, California, cat. no. 237211). ^{32}P-labelled DNA probes specific for 1-SST or 1-FFT were prepared by random oligonucleotide priming and used to screen about 100.000 plaques. Hybridization and washing of Hybond-N membrane were performed under high stringency conditions (hybridization at 65°C, final wash step with 0.1xSSC, 0.1% SDS, 65°C). Positive clones were purified, the pBluescript phagemids excised from the Uni-ZAP vector using the Exassist/Solr system (Stratagene) and the inserts analyzed by restriction enzyme analysis, hybridization

and sequencing.

Analysis of transgenic plants

Analysis of sugars and fructans

50 mg of leaf tissue was frozen in liquid N_2 and homogenized in 0.1 cm^3 Milli Q water in an Eppendorf tube. The homogenate was heated to 90°C for 5 min, then centrifuged at 14,000 g for 10 min. The clear supernatant was analysed by TLC: 2 µl of the supernatant was spotted onto a silica gel G 1500 (Schleicher & Schuell) plate. TLC plates were developed two times with either acetone, water (9:1, v/v), or butan-1-ol, propan-2-ol and water (3:12:4, v/v). Carbohydrates were visualized by spraying the TLC plates with urea-phosphoric acid (Wise et al. 1955).

1-FFT assay

Leaf tissue (50 mg) was frozen in liquid N_2 and homogenized in an Eppendorf tube in 0.1 cm^3 25 mol m^{-3} phosphate (P) buffer, pH 6.5, containing, 2 mol m^{-3} $MgSO_4$, 2 mol m^{-3} Na_2EDTA, 2 mol m^{-3} PMSF (Sigma, USA) 2 mol m^{-3} DTT, 1.5% (w/v) soluble PVPP (Merck) and 20 mol m^{-3} $Na_2S_2O_5$. The homogenate was centrifuged at 14,000 g for 10 min at 4°C. The clear supernatant was used for the 1-FFT assay. Fifty µl of the supernatant was mixed with 50 µl of an assay mixture, containing 2 mol m^{-3} G-$(F)_3$, 80 mol m^{-3} G-$(F)_4$, 50 mol m^{-3} citrate/phosphate buffer, pH 5.5, and 0.02% (w/v) NaN_3. The assay mixture was incubated in the dark at 28°C. Samples of 15 µl were taken after 4, 20, 44 and 68 h of incubation, and analysed by TLC.

EXAMPLES

Example 1. Purification of the sucrose:sucrose fructosyltransferase (1-SST) and fructan fructosyltransferase (1-FFT), and isolation of 1-SST and 1-FFT-encoding cDNAs

1-SST and 1-FFT were purified from tubers of *Helianthus tuberosus* using precipitation techniques, several successive chromatography procedures and electrophoresis. Fractions with

1-SST-activity, eluting from the Mono Q column gave one band after native PAGE. 1-SST is cleaved by SDS, therefore analysis by SDS PAGE yielded two bands of 27 and 55 kDa (Fig. 1, lane 2). Fractions with 1-FFT-activity eluting from the Superdex 75 gel permeation column gave on SDS PAGE one band with an estimated molecular weight of 70 kDa (Fig. 1, lane 3). 1-SST alone was able to synthesize oligofructans from sucrose as a sole substrate (Fig. 3A, 80 h of incubation). By recombining purified 1-SST with purified 1-FFT, sucrose could be converted into fructans with a deree of polymerization of at least 15 [G-$(F)_{14}$, Fig. 3B, 80 h of incubation].

For amino acid sequencing by the Edman (phenylisothiocyanate) degradation method, the 27 kDa (1-SST), 55 kDa (1-SST) and 70 kDa (1-FFT) protein bands were excised from the SDS PAGE gel and subjected to proteolytic digestion by trypsin. The resulting peptide mixtures were separated by RP-HPLC in separate runs (Figs 2A-C). The peptides eluting after 26 min and 37 min (both from the 25 kDa fragment of 1-SST, Fig. 2A), 27 min, 34 min and 37 min (from the 55 kDa fragment of 1-SST, Fig. 2B), 28 min, and 32 min (from 1-FFT, Fig. 2C, the fraction eluting at 32 min contained the peptides 7 and 8) were collected manually and subjected to N-terminal amino acid sequencing (Table 1).

Table 1. Amino acid sequences of selected peptides obtained after tryptic digestion of 1-SST (25 and 55 kDa polypeptides) and 1-FFT, and separation of the resulting peptide mixtures by RP-HPLC.

protein	amino acid sequence	DNA sequence of PCR-primer
1 1-SST,25 kDa	ADVLF??TTSEGSVAR	
2 idem	EQLPVYFYIAK	5'-GARCARYTNCCNGTNTAYTTYTAYATH GCNAAR-3'
3 1-SST,55 kDa	VVLDLETK	
4 idem	FRDPSTLWL?PDGEY	
5 idem	GWANIL	
6 1-FFT	GWATVYNVGR	5'-GGNTGGGCNACNGTNTAYAAY-3'
7 idem	LLVDHSIVEGFAQGGR	5'-ATHGTNGARGGNTTYGCNCAR-3'
8 idem	VGESDS	

Amino acid sequences 2 (1-SST, 25 kDa), 6 and 7 (1-FFT) were used to design DNA primers specific for 1-SST or 1-FFT which were used for PCR. PCR using primers 2, 6 or 7 yielded DNA of about 450, 800 bp and 300 bp, respectively. Nested PCR, using oligonucleotide 7 as specific primer and the 800 bp PCR fragment as template, again yielded the 300 bp PCR fragment which indicated that the 300 bp fragment is included in the 800 bp fragment.

An Uni-ZAP cDNA library constructed from mRNA isolated from *H. tuberosus* tubers was screened with either the 450 bp 1-SST or the 800 bp 1-FFT fragment. Screening of about 100.000 cDNA clones yielded about 20 positive clones hybridizing to the 450 bp fragment and 25 clones hybridizing to the 800 bp fragment. DNA of clones hybridizing to the 450 bp fragment did not hybridize to the 800 bp fragment and vice versa. Positive clones were purified, the pBluescript phagemids excised from the uni-ZAP vector and the insert characterized by restriction enzyme analysis, hybridization and sequencing.

The DNA sequences of 1-SST and 1-FFT and their corresponding amino acid sequences are presented in Fig 4A and Fig 4B, respectively. Sequence ID. No. 1, encoding 1-SST, has an open reading frame of 1890 base pairs and encodes a protein of 630 amino acid residues. On DNA level, 1-SST shows a 68% identity with soluble acid ß-fructofuranosidase (= acid invertase) cDNA from carrot (*Daucus carota*). At the amino acid level, 1-SST shows a 66% similarity with soluble acid ß-fructofuranosidase from carrot.

Sequence ID. No. 2, encoding 1-FFT, has an open reading frame of 1845 base pairs and encodes a protein of 615 amino acid residues and a molecular weight of about 69 kDa. This corresponds to the molecular weight of the purified 1-FFT protein as established by SDS-PAGE (Koops and Jonker, 1994). On DNA level, 1-FFT shows a 65% identity with soluble acid ß-fructofuranosidase (= acid invertase) cDNA from carrot. At the amino acid level, 1-FFT shows a 60% similarity with soluble acid ß-fructofuranosidase from carrot.

Although 1-SST and 1-FFT have a relatively high degree of

homology with acid invertase it has been shown that 1-SST or 1-FFT and invertase are distinctly different enzymes. It has been shown that 1-SST and 1-FFT are unable to catalyse the hydrolysis of sucrose (invertase activity). Hydrolytic activity of purified 1-SST and 1-FFT against sucrose has been tested at a range of pH's and sucrose concentrations. There was no significant invertase activity under any of these conditions (Koops and Jonker 1994). No homology higher than 68% was observed between the DNA sequence encoding 1-SST and any known DNA sequence within the PDB, GENBANK, GENBANK updates, EMBL and EMBL updates nucleotide sequence databases. No homology higher than 65% was observed for the DNA sequence encoding 1-FFT with any known DNA sequence within the PDB, GENBANK, GENBANK updates, EMBL and EMBL updates nucleotide sequence databases.

Example 2. Construction of a chimeric *sst* gene

The full length *sst* cDNA clone, designated pSST 103, was used for the introduction of an NcoI site at the ATG (position 34) and a EcoRV site downstream of the stopcodon (at position 1924) using PCR. From the plasmid pMOG18 (Pen et al., 1992) which contains the enhanced CaMV35S promoter, ALMV leader sequence, *uidA* gene and the *nos* terminator sequence, the *uidA* coding sequence was replaced by the *sst* cDNA. pMOG18 was digested with BamHI, filled in with Klenow DNA polymerase, and digested with NcoI. The *sst* PCR fragment, cut with NcoI and EcoRI, was ligated into this vector, resulting in the clone pSST217. The EcoRI/HindII fragment of pSST217 containing the complete chimeric construct (enh.35S+ALMV-*sst*-*nos*) was cloned into the EcoRI and HindIII site of pBINPLUS (Van Engelen et al., 1995), a binary plant transformation vector derived from pBIN19 (Bevan, 1984) resulting in plasmid pVS1 (Fig. 5A).

Example 3. Construction of a chimeric *fft* gene

The full length *fft* cDNA clone, designated pFFT 111, was used for the introduction of an NcoI site at the ATG (position 29) and a BamHI site downstream of the stopcodon (at position

1874) using PCR. From the plasmid pMOG18 (Pen et al., 1992) which contains the enhanced CaMV35S promoter, ALMV leader sequence, *uidA* gene and the *nos* terminator sequence, the *uidA* coding sequence was replaced by the *fft* cDNA. The PCR fragment digested with NcoI and BamHI was ligated into the pMOG18 vector digested with NcoI and BamHI, resulting in clone pFFT209. The HindIII/EcoRI fragment of pFFT209 containing the complete chimeric construct (enh.35S+ALMV-*fft*-*nos*) was cloned into the HindIII and EcoRI site of pBINPLUS (Van Engelen et al., 1995), a binary plant transformation vector derived from pBIN19 (Bevan, 1984) resulting in plasmid pVF1 (Fig. 5B).

Example 4. Transformation of Petunia and potato plants

The binary vectors pVS1 and pVF1 (Fig. 5) were conjugated from *E. coli* XL1-Blue to *Agrobacterium tumefaciens* strain AGLO by triparental mating (Ditta et al., 1980). Exconjugants were used to transform *Petunia hybrida* leaf discs as described by Horsch et al. (1985). Leaf discs were prepared from top leaves of young non-flowering plants. *P. hybrida* variety W115 was used for the transformation experiments. Exconjugants were also used to transform diploid potato (*Solanum tuberosum*, variety Kardal) stem explants as described previously (Visser, 1991). After shoot and root regeneration on kanamycin-containing media, plants were put in soil and transferred to the greenhouse. Plants regenerated (on kanamycin-free media) from leaf discs and stem explants treated with the Agrobacterium strain AGLO lacking a binary vector served as a control.

Example 5. Analysis of transgenic plants expressing the *sst* and *fft* genes

About 25 transgenic *petunia* plants and 25 transgenic potato plants were generated harboring the pVS1 construct and 25 transgenic *petunia* and potato plants harboring the pVF1 construct. Ten *petunia* and ten potato plants were transformed with the Agrobacterium strain AGL0 lacking a binary vector. These plants were used as a control. Southern blot analysis of

genomic DNA isolated from the transformed plants showed that on average 1-5 copies of the introduced chimeric genes were integrated (data not shown).

The carbohydrate composition of transgenic plants was analysed by two essentially different techniques: thin layer chromatography (TLC), which separate carbohydrates on basis of liquid-liquid partitioning (after TLC, the fructans were detected by a fructose specific colour reaction) and HPAEC, which separate carbohydrates at alkaline conditions (pH 13) on basis of charge, and detect carbohydrates by oxidation with a gold working electrode. Analysis of leaves extracts from the potato and *Petunia* plants harboring the pVS1 construct showed that both transgenic plant species contain products which are the result of SST activity. TLC showed the presence of at least the trisaccharide G-$(F)_2$ and most probably also the tetrasaccharide G-$(F)_3$ and the pentasaccharide G-$(F)_4$ in extracts of potato leaves, whereas these oligofructans were absent in the control plant (Fig. 6). The presence of G-$(F)_2$ and G-$(F)_3$ in leave extracts of potato but also of transgenic *P. hybrida* plants was demonstrated by HPAE analysis (Figs. 7 and 8). HPAEC, which is more sensitive and more specific than TLC, also revealed a small amount of G-$(F)_4$ in transgenic potato (Fig. 8). The results of Figs 6-8 clearly indicate that the *sst* gene is expressed into an enzymatically active SST protein in both *P. hybrida* and potato.

Transgenic plants harboring the pVF1 construct did not contain fructans because FFT needs oligofructans (such as G-$(F)_2$ or G-$(F)_3$) as initial substrates for the synthesis of fructans. Oligofructans are not present in plants lacking SST activity such as wild type potato or *Petunia*, or transgenic plants only containing the pVF1 construct. The presence of FFT activity in transgenic plants harboring the pVF1 construct is therefore verified by FFT activity measurements. The FFT assay used to evaluate the presence of an active FFT in transgenic potato was based on the ability of FFT to catalyse the synthesis of G-$(F)_n$, n>4, at the expense of G-$(F)_3$. The total leaf protein extract from potato plants harboring the pVF1

construct, was mixed with an G-$(F)_4$ containing assay mixture, and the presence of fructans with a higher degree of polymerization (G-$(F)_n$, n>4) was examined by TLC. Already after 4 h of incubation, G-$(F)_5$ and G-$(F)_6$ could be detected. After 44 h of incubation, fructans with a degree of polymerization higher then 4, including G-$(F)_4$, G-$(F)_5$, G-$(F)_6$, G-$(F)_7$ and G-$(F)_8$, could be detected, whereas these fructans were absent in the control mixtures (Fig. 9). This indicates that also the *fft* gene is expressed into an enzymatically active FFT protein.

DESCRIPTION OF THE FIGURES

Fig. 1. Analysis of Mono Q fractions of 1-SST purification and Superdex HR 75 fractions of 1-FFT purification on SDS-PAGE. Lane 1, Molecular Weight marker (MW is given in kDa); lane 2, Mono Q fraction with 1-SST activity; lane 3, Superdex HR 75 fractions with 1-FFT activity.

Fig. 2. RP-HPLC separations of tryptic digests of: a. the 25 kDa polypeptide of 1-SST (A); b. the 55 kDa polypeptide of 1-SST (B); c. the 70 kDa 1-FFT polypeptide (C). Free eluting peptide fractions indicated with arrows were collected manually and subjected to amino acid sequencing.

Fig. 3. HPAEC separations of oligofructans synthesized from sucrose by purified 1-SST (A) and by a mixture of purified 1-SST and purified 1-FFT (B). The reactions were performed in 100 mol m^{-3} sucrose, 2 mol m^{-3} DTT, 10 mol m^{-3} citrate/phosphate buffer, pH 5.0, 0.01% Na-azide at 25°C. Reaction time was 80 h. Reaction was stopped by boiling the reaction mixture for 5 min. Rhamnose was used as an internal standard.

Fig. 4. Nucleotide sequence and deduced amino acid sequence of the isolated 1-SST (A) and 1-FFT (B) cDNA. The amino acids determined by amino acid sequencing (see also Table 1) of the purified 1-SST and 1-FFT proteins are underlined.

Fig. 5. Gene constructs pVS1 (A) and pVF1 (B). The chimeric constructs consist of the enhanced CaMV35S promoter with ALMV translational enhancer (X), the coding sequence of *sst* (Y) or *fft* (W), and the *nos* termination signal (Z). Restriction sites

which were used in the cloning procedure are indicated.

Fig. 6. TLC analysis of fructans in transgenic potato plants harboring the pSF1 construct. TLC plates were developed twice in 90% aqueous acetone. S=sucrose standard, G=glucose standard, F=fructose standard, H=standard fructan mixture from tubers of *H. tuberosus*, N=Neosugar standard, K=G-$(F)_2$-standard. No 1-6 represent individual potato plants harboring the pVS1 construct. C is control plant harboring the AGL0 construct.

Fig. 7. HPAEC separations of carbohydrates extracted from leaves of *Petunia hybrida* harboring the pVS1 construct (a), a standard fructan mixture extracted from *H. tuberosus* tubers (b), and carbohydrates from leaves of the control *Petunia* harboring the AGL0 construct (c).

Fig. 8. HPAEC separations of carbohydrates extracted from leaves of potato (*Solanum tuberosum*) harboring the pVS1 construct (a), a standard fructan mixture extracted from *H. tuberosus* tubers (b), and carbohydrates from leaves of the control potato harboring the AGL0 construct (c).

Fig. 9. TLC analysis of fructans synthesised from G-(F), by protein extracts from leaves of transgenic potato plants harboring the pVF1 construct. TLC plates were developed twice in butan-1-ol, propan-2-ol and water (3:12:4, v/v). S=sucrose standard; N=Neosugar standard, H=standard fructan mixture from tubers of *H. tuberosus*. No 1-8 represent different individual transgenic potato plants harboring the pVF1 construct. C_1 and C_2 are control plants harboring the AGL0 construct.

DEFINITIONS AND ABBREVIATIONS

Fructan nomenclature is as according to Lewis (1993).

Fructosyl unit: a fructose molecule linked to another sugar molecule (e.g. glucose, fructose or galactose). Abbreviated as -F (e.g. in G-F) or -F- (e.g. G-F-F). For molecules consisting of more then one fructosyl unit (e.g. G-F-F-F-F-F-F) a condensed notation is used [G-$(F)_6$] or GF_6. G-$(F)_6$ consists of one glucosyl and 6 fructosyl units. G-$(F)_6$ consists of one

fructosyl-glucose linkage and 5 fructosyl-fructose linkages.

Oligofructans: Any compound with one, two, or three fructosyl-fructose linkages (a glucose may be present but is not necessary). In the present application, the term oligofructans was used to denote the products of SST-activity [G-$(F)_2$, G-$(F)_3$ and G-$(F)_4$]. Oligofructans are more generally denoted as short chain fructans or fructans with a low degree of polymerization. In the present application oligofructans also include compounds consisting of 2, 3 or 4 fructosyl units, but lacking the glucosyl unit.

Fructans: Any compound in which one or more fructosyl-fructose linkages constitute a majority of the linkages (a glucose may be present but is not necessary). Fructan is used as a numerative noun where the materials referred to are chemically distinct (e.g. the fructans G-$(F)_2$ and G-$(F)_{13}$. In the present application, fructans are defined as the products of FFT activity (G-F_n, $2 \leq n < \pm 60$). Unless indicated otherwise, fructans include also oligofructans. To denote a restricted group of fructans the "G-F_n, n=.." notation is used. In the present application fructans also include compounds consisting of more than 1 fructosyl unit, but lacking the glucosyl unit.

Inulin: Fructan that has mostly the ß-2,1-fructosyl-fructose linkage (a glucose may be present, but not is necessary). Cumulative vs. numerative usage as a noun is the same as for fructan above.

Levan: Fructan that has mostly the ß-2,1-fructosyl-fructose linkage (a glucose is allowed but not necessary). Levan is also used to denote fructans from bacterial origin, although bacterial fructans do not always consists of predominantly ß-2,6 fructosyl-fructose linkages. For example, the fructans synthesized by levansucrase from *Bacillus subtilus* have predominantly ß-2,1-fructosyl-fructose linkages (inulin). Cumulative vs. numerative usage of levan as a noun is the same as for fructan.

Levansucrase: Enzymes from bacterial origin that are involved in the synthesis of fructan.

Sucrose:sucrose fructosyltransferase (SST): Plant-derived

enzyme catalyzing the initial step of fructan synthesis (reaction 2). Enzyme can also be involved in the synthesis of oligofructans [G-$(F)_n$, $2 \leq n \leq 4$] (reaction 3). In the present application, the designation SST can include either 1-SST, an SST form involved in the biosynthesis of oligofructans that has mostly the ß-2,1-fructosyl-fructose linkage; or 6-SST, an SST form involved in the biosynthesis of oligofructans that has mostly the ß-2,6-fructosyl-fructose linkage; or 1-SST and 6-SST.

Fructan:fructan fructosyltransferase (FFT): Plant-derived enzyme involved in the synthesis of fructans. Enzyme capable of catalyzing the synthesis of oligofructans and fructans of a higher degree of polymerization. FFT from *H. tuberosus* has overlapping activity with SST from *H. tuberosus* (reaction 3), but cannot catalyse the initial step of fructan synthesis (reaction 2). In the present application, the designation FFT can include either 1-FFT, an FFT form involved in the biosynthesis of oligofructans that has mostly the ß-2,1-fructosyl-fructose linkage; or 6-FFT, an FFT form involved in the biosynthesis of fructans that has mostly the ß-2,6-fructosyl-fructose linkage; or 1-FFT and 6-FFT.

Invertase: ß-fructosidase or ß-fructofuranosidase.

Degree of polymerization (DP): term to indicate the total amount of fructosyl and glycosyl residues; for example, G-$(F)_2$ has a DP of 3. The n-value in G-F_n increases with an increasing degree of polymerization.

Fructan profile: term to describe the fructan size/distribution pattern, or alternatively, the relative amounts and kinds of fructans, in an extract for example derived from a plant, plant organ or plant cell. The currently most reliable method to analyze a fructan pattern of an extract is by high performance anion exchange chromatography and pulsed amperometric detection (Chatterton et al. 1990).

Helianthus tuberosus: Jerusalem artichoke.

Cichorium intybus: chicory.

HPLC: High Performance Liquid Chromatography. Technique for the separation of complex mixtures of compounds. Variants on

this technique are high performance reversed phase chromatography (*RP HPLC*) or high performance anion exchange chromatography in combination with pulsed amperometric detection (*HPAEC-PAD*, see for example Fig. 3).

REFERENCES

-Angenent GC, Ebskamp MJM, Weisbeek PJ and Smeekens SCM. (1993). Purification and properties of sucrose-sucrose fructosyltransferases in barley leaves and onion seeds. In: Fuchs A, ed. Inulin ans inulin containing crops. Elsevier, Amsterdam, the Netherlands, pp. 173-184.

-Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K. (1994).Current protocols in molecular biology. John Wiley & Sons.

-Bevan M. (1984). Binary *Agrobacterium* vectors for plant transformation. Nucleic Acids Research 12, 8711-8721.

-Carlsson J. (1970). A levansucrase from *Streptococcus mutans*. Caries Research 4, 97-113

-Chatterton NJ, Harrison PA, Thornley WR, Draper EA. 1990. Oligosaccharides in foliage of *Agropyron*, *Bromus*, *Dactylis*, *Festuca*, *Lolium*, and *Phleum*. New Phytologist 114, 167-171.

-Darwen CWE, John P. (1989). Localization of the enzymes of fructan metabolism in vacuoles isolated by a mechanical method from tubers of Jerusalem artichoke (*Helianthus tuberosus* L.). Plant Physiology 89, 658-663.

-Dedonder R. (1966). Levansucrase from *Bacillus subtilus*. Methods in Enzymology 8, 500-505.

-Ditta G, Stanfield S, Corbi D. and Helinski DR (1980). Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proceedings National Academy Science USA 77, 7347-7351.

-Ebskamp MJM, Van der Meer IM, Spronk BA, Weisbeek PJ and Smeekens JCM (1994). Accumulation of fructose polymers in transgenic tabacco. Bio/Technology 12, 272-275.

-Heinze B and Praznik W. (1991). Separation and purification of inulin oligomers and polymers by reversed-phase high-performance liquid chromatography. Journal of Applied Polymer Science: Applied Polymer Symposium 48, 207-225.

-Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG and Fraley RT. (1985). A simple and general method for transferring genes into plants. Science 227, 1229-1232.

-Jacques NA. (1993). The fructosyltransferase of *Streptococcus*

salivarius. New Phytologist 123, 429-435.

-Koops AJ and Jonker HH. (1994). Purification and characterization of the enzymes of fructan biosynthesis in tubers of *Helianthus tuberosus* 'Colombia'. I. Fructan:fructan fructosyltransferase. Journal of Experimental Botany 45, 1623-1631

-Laemmli UK. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227, 680-685.

-Lewis DH. (1993). Nomenclature and diagrammatic representation of oligomeric fructans - a paper for discussion New Phytologist. 124, 583-594.

-Lüscher M, Frehner M, Nösberger J. (1993). Purification and characterization of fructan:fructan fructosyltransferase from Jerusalem artichoke (*Helianthus tuberosus* L.). New Phytologist 123, 717-24.

-Meier H, Reid JSG. (1982). Reserve polysaccharides other than starch in higher plants. In: Loewus FA, Tanner W, eds. Encyclopedia of Plant Physiology, New Series 13. Berlin: Springer Verlag, 1418-71.

-Pen J, Molendijk L, Quax WJ, Sijmons PC, van Ooyen AJJ, van den Elzen PJM, Rietveld K. and Hoekema A. (1992). Production of active *Bacillus licheniformis* alpha-amylase in tobacco and its application in starch liquefaction. Bio/Technology 10, 292-296.

-Praznik W, Beck RHF and Spies Th. (1990). Isolation and characterization of sucrose:sucrose 1^F-β-D-fructosyltransferase from tubers of *Helianthus tuberosis*, L. Agric. biol. Chem. 54, 2429-2431.

-Rosenfeld J, Capdevielle J, Guillemot JC, Ferrara P. (1992). In-gel digestion of proteins for internal sequence analysis after one- or two-dimensional gel electrophoresis. Analytical Biochemistry 203, 173-179.

-Sambrook J, Fritsch EF, Maniatis T. (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

-Sato S, Kuramitsu HK. (1986). Isolation and characterization of a fructosyltransferase gene from *Streptococcus mutans* GS-5.

Infect. Immun 52, 166-170.

-Shiomi N and Izawa M. (1980). Purification and characterization of sucrose:sucrose 1-fructosyltransferase from roots of asparagus (*Asparagus officinalis* L.). Agric. Biol. Chem. 44, 603-614.

-Steinmetz M, Le Coq D, Aymerich S, Gonzy Treboul G, Gay P. (1985). The DNA sequence of the gene for the secreted *Bacillus subtilus* enzyme levansucrase and its genetic control sites. Molecular & General Genetics 200, 220-228.

-Van der Meer IM, Ebskamp MJM, Visser RGF, Weisbeek PJ and Smeekens JCM. (1994). Fructan as a new carbohydrate sink in transgenic potato plants. Plant Cell 6, 561-570.

-Van Engelen FA, Molthoff JW, Conner, AJ, Nap J-P, Pereira A, and Stiekema WJ. (1995). pBINPLUS: an improved plant transformation vector based on pBIN19. Transgenic Research 4, 288-290.

-Visser RGF. (1991). Regeneration and transformation of potato by *Agrobacterium tumefaciens*. In: *Plant Tissue Culture Manual B5*, edited by Lindsey, K. Dordrecht, The Netherlands, Kluwer Academic Publishers, pp. 1-9.

-Wagner W, Keller F, Wiemken. (1983). Fructan metabolism in cereals: induction in leaves and compartmentation in protoplasts and vacuoles. Zeitschrift für Pflanzenphysiologie 112, 359-372.

-Wise CS, Dimler RJ, Davi9s HA, Rist CE. 1955. Determination of easily hydrolysable fructose units in dextran preparations. Analytical Chemistry 27, 33-6.

CLAIMS

1. A DNA fragment having a nucleotide sequence SEQ ID No.1 as shown in Fig. 4A or a homologous sequence having a similarity of at least 70% encoding 1-sucrose:sucrose fructosyltransferase.

2. A DNA fragment having a nucleotide sequence SEQ ID No. 2 as shown in Fig. 4B or a homologous sequence having a similarity of at least 70% encoding 1-fructan:fructan fructosyltransferase.

3. A DNA fragment according to claims 1 or 2 having a similarity of at least 85%.

4. A recombinant DNA sequence comprising one or more DNA fragments as defined in claims 1 to 3.

5. A recombinant DNA sequence comprising one or more DNA fragments as defined in claims 1 to 3, said fragment(s) being present in the inverted orientation.

6. A method for producing a genetically transformed host organism showing a modified fructan profile, which comprises the steps of:
i) preparing a chimeric gene construct comprising one or more DNA fragments as defined in claims 1 to 3, or said DNA fragments in the inverted orientation, operably linked to a promotor sequence active in said host organism and a terminator sequence active in said host organism,
ii) introducing the chimeric gene construct into the genome of the host organism.

7. A method according to claim 6, wherein said host organism is a plant, and the method additionally comprises the step of
iii) regenerating the transformed plant cells to transgenic plants.

8. A transformed plant produced by the method of claim 7, or a plant cell, seed, fruit, seedling or any plant part harboring said chimeric gene construct.

5

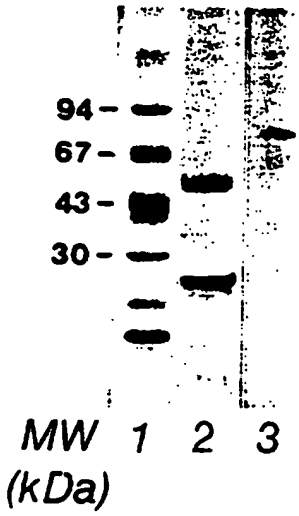


Fig. 1.

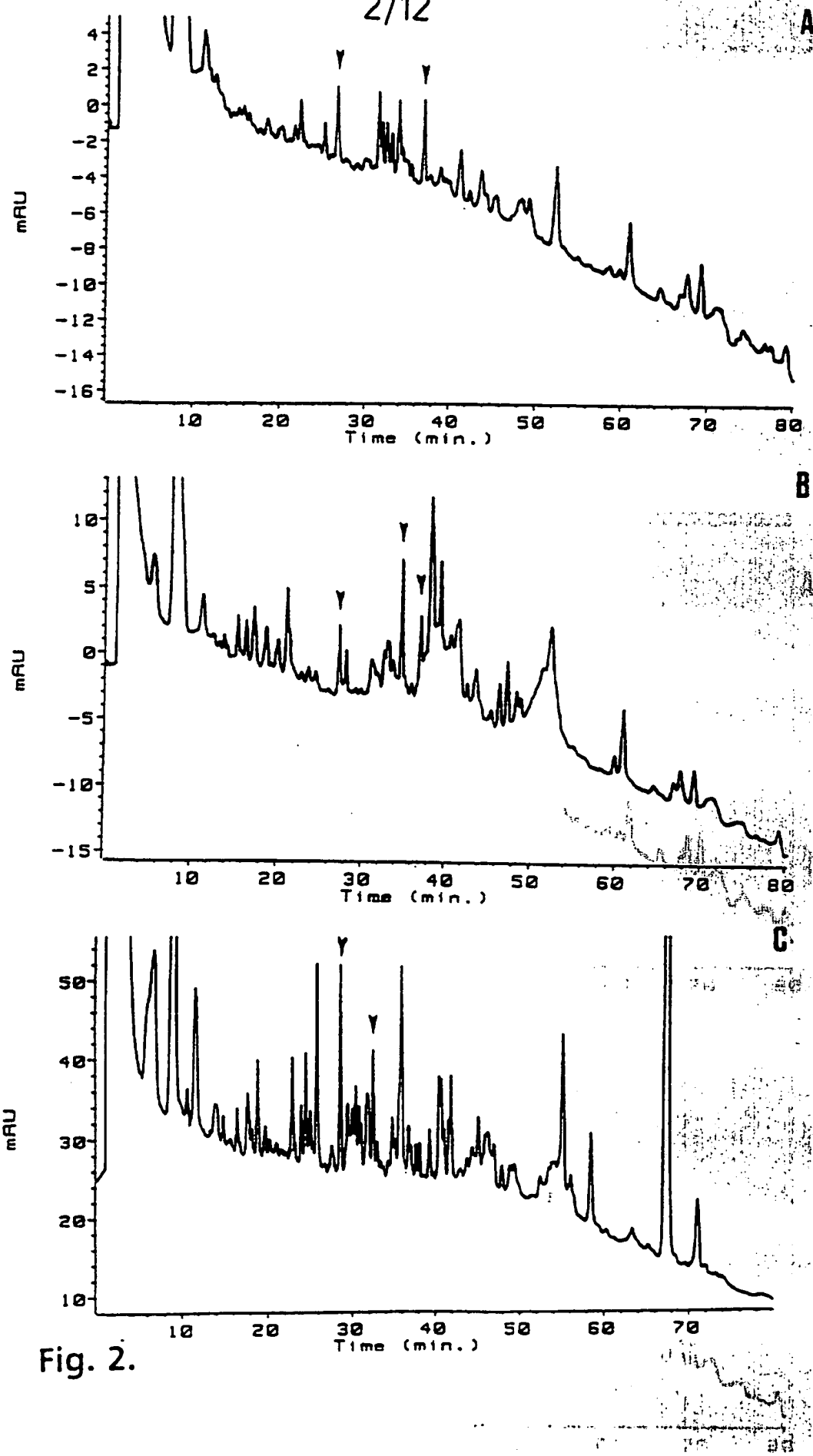

A
mAU
4
2
0
-2
-4
-6
-8
-10
-12
-14
-16
10
20
30
40
50
60
70
80
Time (min.)
B
mAU
10
5
0
-5
-10
-15
10
20
30
40
50
60
70
80
Time (min.)
C
mAU
50
40
30
20
10
10
20
30
40
50
60
70
Time (min.)

Fig. 2.

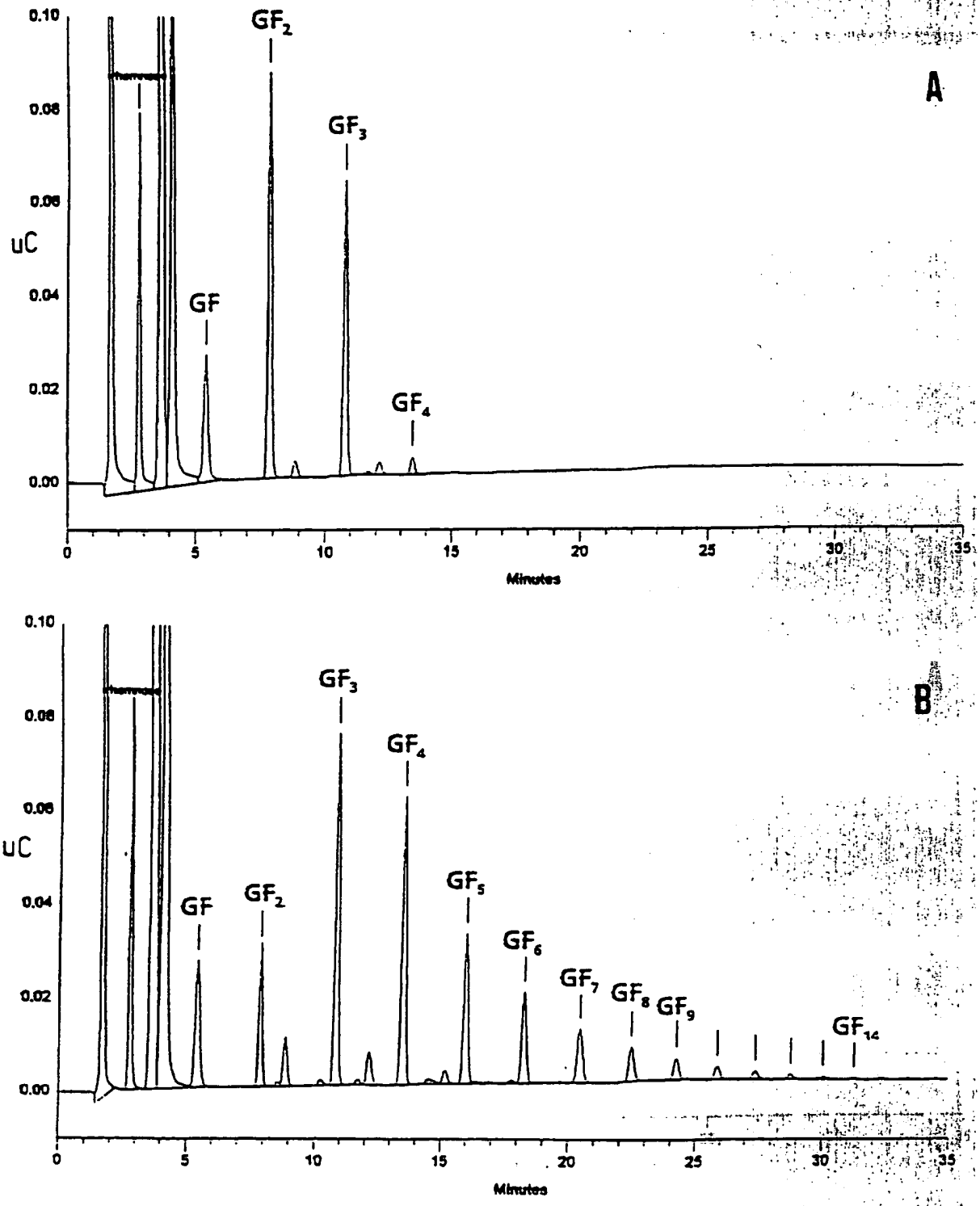


Fig. 3.

A. SEQ ID. NO. 1. 4/12

FIG. 4

```
GGCACGAGAAAAAACCCTCCCTCAGGCCACCACATGATGGCTTCATCCACCACCACCACC 60
                                    M  M  A  S  S  T  T  T  T
CCTCTCATTCTCCATGATGACCCTGAAAACCTCCCAGAACTCACCGGTTCTCCGACAACT 120
 P  L  I  L  H  D  D  P  E  N  L  P  E  L  T  G  S  P  T  T
CGTCGTCTATCCATCGCAAAAGTGCTTTCGGGGATCCTTGTTTCGGTTCTGGTTATAGGT 180
 R  R  L  S  I  A  K  V  L  S  G  I  L  V  S  V  L  V  I  G
GCTCTTGTTGCTTTAATCAACAACCAAACATATGAATCCCCCTCGGCCACCACATTCGTA 240
 A  L  V  A  L  I  N  N  Q  T  Y  E  S  P  S  A  T  T  F  V
ACTCAGTTGCCAAATATTGATCTGAAGCGGGTTCCAGGAAAGTTGGATTCGAGTGCTGAG 300
 T  Q  L  P  N  I  D  L  K  R  V  P  G  K  L  D  S  S  A  E
GTTGAATGGCAACGATCCACTTATCATTTTCAACCCGACAAAAATTTCATTAGCGATCCT 360
 V  E  W  Q  R  S  T  Y  H  F  Q  P  D  K  N  F  I  S  D  P
GATGGCCCAATGTATCACATGGGATGGTATCATCTATTTTATCAGTACAACCCTCAATCT 420
 D  G  P  M  Y  H  M  G  W  Y  H  L  F  Y  Q  Y  N  P  Q  S
GCCATCTGGGGCAACATCACATGGGGCCACTCGGTATCGAAAGACATGATCAACTGGTTC 480
 A  I  W  G  N  I  T  W  G  H  S  V  S  K  D  M  I  N  W  F
CATCTCCCTTTCGCCATGGTTCCTGACCATTGGTACGACATCGAAGGTGTCATGACGGGT 540
 H  L  P  F  A  M  V  P  D  H  W  Y  D  I  E  G  V  M  T  G
TCGGCTACAGTCCTCCCTAATGGTCAAATCATCATGCTTTACTCGGGCAACGCGTATGAT 600
 S  A  T  V  L  P  N  G  Q  I  I  M  L  Y  S  G  N  A  Y  D
CTCTCCCAAGTACAATGCTTGGCGTACGCTGTCAACTCGTCGGATCCACTTCTTATAGAG 660
 L  S  Q  V  Q  C  L  A  Y  A  V  N  S  S  D  P  L  L  I  E
TGGAAAAAATATGAAGGTAACCCTGTCTTACTCCCACCACCAGGAGTAGGCTACAAGGAC 720
 W  K  K  Y  E  G  N  P  V  L  L  P  P  P  G  V  G  Y  K  D
TTTCGGGACCCATCCACATTGTGGTCGGGCCCTGATGGTGAATATAGAATGGTAATGGGG 780
 F  R  D  P  S  T  L  W  S  G  P  D  G  E  Y  R  M  V  M  G
TCCAAGCACAACGAGACTATTGGCTGTGCTTTGATTTACCATACCACTAATTTTACGCAT 840
 S  K  H  N  E  T  I  G  C  A  L  I  Y  H  T  T  N  F  T  H
TTTGAATTGAAAGAGGAGGTGCTTCATGCAGTCCCACATACTGGTATGTGGGAATGTGTT 900
 F  E  L  K  E  E  V  L  H  A  V  P  H  T  G  M  W  E  C  V
GATCTTTACCCGGTGTCCACCGTACACACAAACGGGCTGGACATGGTGGATAACGGGCCA 960
 D  L  Y  P  V  S  T  V  H  T  N  G  L  D  M  V  D  N  G  P
AATGTTAAGTACGTGTTGAAACAAAGTGGGGATGAAGATCGCCATGATTGGTATGCAATT 1020
 N  V  K  Y  V  L  K  Q  S  G  D  E  D  R  H  D  W  Y  A  I
GGAAGTTACGATATAGTGAATGATAAGTGGTACCCAGATGACCCGGAAAATGATGTGGGT 1080
 G  S  Y  D  I  V  N  D  K  W  Y  P  D  D  P  E  N  D  V  G
ATCGGATTAAGATATGATTTTGGAAAATTTTATGCGTCCAAGACGTTTTATGACCAACAT 1140
 I  G  L  R  Y  D  F  G  K  F  Y  A  S  K  T  F  Y  D  Q  H
AAGAAGAGGAGAGTCCTTTGGGGCTATGTTGGAGAAACCGATCCCCAAAAGTATGACCTT 1200
 K  K  R  R  V  L  W  G  Y  V  G  E  T  D  P  Q  K  Y  D  L
TCAAAGGGATGGGCTAACATTTTGAATATTCCAAGGACCGTCGTTTTGGACCTCGAAACT 1260
 S  K  G  W  A  N  I  L  N  I  P  R  T  V  V  L  D  L  E  T
```

FIG. 4

```
AAAACCAATTTGATTCAATGGCCAATCGAGGAAACCGAAAACCTTAGGTCGAAAAAGTAT 1320
 K  T  N  L  I  Q  W  P  I  E  E  T  E  N  L  R  S  K  K  Y
GATGAATTTAAAGACGTCGAGCTTCGACCCGGGGCACTCGTTCCCCTTGAGATAGGCACA 1380
 D  E  F  K  D  V  E  L  R  P  G  A  L  V  P  L  E  I  G  T
GCCACACAGTTGGATATAGTTGCGACATTCGAAATCGACCAAAAGATGTTGGAATCAACG 1460
 A  T  Q  L  D  I  V  A  T  F  E  I  D  Q  K  M  L  E  S  T
CTAGAGGCCGATGTTCTATTCAATTGCACGACTAGTGAAGGCTCGGTTGCAAGGAGTGTG 1500
 L  E  A  D  V  L  F  N  C  T  T  S  E  G  S  V  A  R  S  V
TTGGGACCGTTTGGTGTGGTGGTTCTAGCCGATGCCCAGCGCTCCGAACAACTTCCTGTA 1560
 L  G  P  F  G  V  V  V  L  A  D  A  Q  R  S  E  Q  L  P  V
TACTTCTATATCGCAAAAGATATTGATGGAACCTCACGAACTTATTTTTGTGCCGACGAA 1620
 Y  F  Y  I  A  K  D  I  D  G  T  S  R  T  Y  F  C  A  D  E
ACAAGATCATCCAAGGATGTAAGCGTAGGGAAATGGGTGTACGGAAGCAGTGTTCCTGTC 1680
 T  R  S  S  K  D  V  S  V  G  K  W  V  Y  G  S  S  V  P  V
CTCCCAGGCGAAAAGTACAATATGAGGTTATTGGTGGATCATTCGATAGTAGAGGGATTT 1740
 L  P  G  E  K  Y  N  M  R  L  L  V  D  H  S  I  V  E  G  F
GCACAAAACGGGAGAACCGTGGTGACATCAAGAGTGTATCCAACAAAGGCGATCTACAAC 1800
 A  Q  N  G  R  T  V  V  T  S  R  V  Y  P  T  K  A  I  Y  N
GCTGCGAAGGTGTTTTTGTTCAACAACGCGACTGGAATCAGTGTGAAGGCGTCGATCAAG 1860
 A  A  K  V  F  L  F  N  N  A  T  G  I  S  V  K  A  S  I  K
ATCTGGAAGATGGGGGAAGCAGAACTCAATCCTTTCCCTCTTCCTGGGTGGACTTTCGAA 1920
 I  W  K  M  G  E  A  E  L  N  P  F  P  L  P  G  W  T  F  E
CTTTGATGGTTATATTTTGGACCCTATATATGTGTTATTATCATGATGGTTATATTTTGG 1980
 L
ACCCTATATATGTGTTATTATCATGAAGCATAAGTTTGGACTGGAGGGGGTATTATTGTA 2040
ATTTTATATGCATGTTCTATTACTTGTGAGGTTATAGTATGTAATTAAATTATTATATAC 2100
TATATCAATTTCTAAT                                               2116
```

B. SEQ ID. NO. 2. 6/12

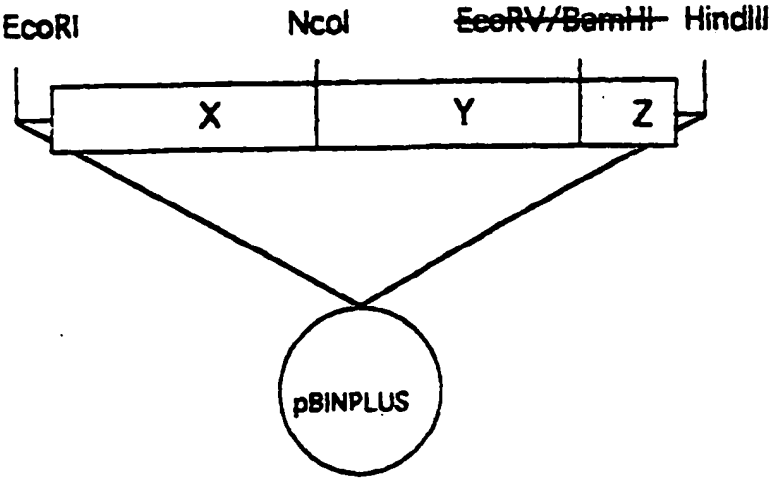
FIG. 4

```
GGGACGAGTACCAGTCCAGTCAGTCACCATGCAAACCCCTGAACCCTTTACAGACCTTGA 60
                                M  Q  T  P  E  P  F  T  D  L  E
ACATGAACCCCACACACCCCTACTGGACCACCACCACAACCCACCACCACAAACCACCAC 120
  H  E  P  H  T  P  L  L  D  H  H  H  N  P  P  P  Q  T  T  T
AAAACCTTTGTTCACCAGGGTTGTGTCCGGTGTCACCTTTGTTTTATTCTTCTTTGGTTT 180
  K  P  L  F  T  R  V  V  S  G  V  T  F  V  L  F  F  F  G  F
CGCTATCGTATTCATTGTTCTCAACCAACAGAATTCTTCTGTTCGTATCGTCACCAATTC 240
  A  I  V  F  I  V  L  N  Q  Q  N  S  S  V  R  I  V  T  N  S
GGAGAAATCTTTTATAAGGTATTCGCAGACCGATCGCTTGTCGTGGGAACGGACCGCTTT 300
  E  K  S  F  I  R  Y  S  Q  T  D  R  L  S  W  E  R  T  A  F
TCATTTTCAGCCTGCCAAGAATTTTATTTACGATCCAGATGGTCAGTTGTTTCACATGGG 360
  H  F  Q  P  A  K  N  F  I  Y  D  P  D  G  Q  L  F  H  M  G
CTGGTACCATATGTTCTATCAATACAACCCATACGCACCGGTTTGGGGCAATATGTCATG 420
  W  Y  H  M  F  Y  Q  Y  N  P  Y  A  P  V  W  G  N  M  S  W
GGGTCACTCAGTGTCCAAAGACATGATCAACTGGTACGAGCTGCCAGTCGCTATGGTCCC 480
  G  H  S  V  S  K  D  M  I  N  W  Y  E  L  P  V  A  M  V  P
GACCGAATGGTATGATATCGAGGGCGTCTTATCCGGGTCTACCACGGTCCTTCCAAACGG 540
  T  E  W  Y  D  I  E  G  V  L  S  G  S  T  T  V  L  P  N  G
TCAGATCTTTGCATTGTATACTGGGAACGCTAATGATTTTTCCCAATTACAATGCAAAGC 600
  Q  I  F  A  L  Y  T  G  N  A  N  D  F  S  Q  L  Q  C  K  A
TGTACCCGTAAACTTATCTGACCCGCTTCTTATTGAGTGGGTCAAGTATGAGGATAACCC 660
  V  P  V  N  L  S  D  P  L  L  I  E  W  V  K  Y  E  D  N  P
AATCCTGTACACTCCACCAGGGATTGGGTTAAAGGACTATCGGGACCCGTCAACAGTCTG 720
  I  L  Y  T  P  P  G  I  G  L  K  D  Y  R  D  P  S  T  V  W
GACAGGTCCCGATGGAAAGCATAGGATGATCATGGGAACTAAACGTGGCAATACAGGCAT 780
  T  G  P  D  G  K  H  R  M  I  M  G  T  K  R  G  N  T  G  M
GGTACTTGTTTACTATACCACTGATTACACGAACTACGAGTTGTTGGATGAGCCGTTGCA 840
  V  L  V  Y  Y  T  T  D  Y  T  N  Y  E  L  L  D  E  P  L  H
CTCTGTTCCCAACACCGATATGTGGGAATGCGTCGACTTTTACCCGGTTTCGTTAACCAA 900
  S  V  P  N  T  D  M  W  E  C  V  D  F  Y  P  V  S  L  T  N
TGATAGTGCACTTGATATGGCGGCCTATGGGTCGGGTATCAAACACGTTATTAAAGAAAG 960
  D  S  A  L  D  M  A  A  Y  G  S  G  I  K  H  V  I  K  E  S
TTGGGAGGGACATGGAATGGATTGGTATTCAATCGGGACATATGACGCGATAAATGATAA 1020
  W  E  G  H  G  M  D  W  Y  S  I  G  T  Y  D  A  I  N  D  K
ATGGACTCCCGATAACCCGGAACTAGATGTCGGTATCGGGTTACGGTGCGATTACGGGAG 1080
  W  T  P  D  N  P  E  L  D  V  G  I  G  L  R  C  D  Y  G  R
```

FIG.4

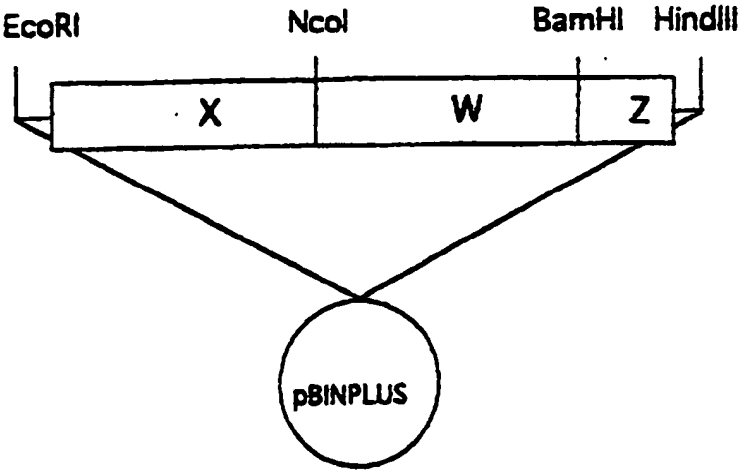
```
GTTTTTTGCATCAAAGAGTCTTTATGACCCATTGAAGAAAAGGAGGATCACTTGGGGTTA 1140
  F  F  A  S  K  S  L  Y  D  P  L  K  K  R  R  I  T  W  G  Y
TGTTGGAGAATCAGATAGTGCTGATCAGGACCTCTCTAGAGGATGGGCTACTGTTTATAA 1200
  V  G  E  S  D  S  A  D  Q  D  L  S  R  G  W  A  T  V  Y  N
TGTTGGAAGAACAATTGTACTAGATAGAAAGACCGGGACCCATTTACTTCATTGGCCCGT 1260
  V  G  R  T  I  V  L  D  R  K  T  G  T  H  L  L  H  W  P  V
TGAGGAAGTCGAGAGTTTGAGATACAACGGTCAGGAGTTTAAAGAGATCAAGCTAGAGCC 1320
  E  E  V  E  S  L  R  Y  N  G  Q  E  F  K  E  I  K  L  E  P
CGGTTCAATCATTCCACTCGACATAGGCACGGCTACACAGTTGGACATAGTTGCAACATT 1380
  G  S  I  I  P  L  D  I  G  T  A  T  Q  L  D  I  V  A  T  F
TGAGGTGGATCAAGCAGCGTTGAACGCGACAAGTGAAACCGATGATATTTATGGTTGCAC 1440
  E  V  D  Q  A  A  L  N  A  T  S  E  T  D  D  I  Y  G  C  T
CACTAGCTTAGGTGCAGCCCAAAGGGGAAGTTTGGGACCATTTGGTCTTGCGGTTCTAGC 1500
  T  S  L  G  A  A  Q  R  G  S  L  G  P  F  G  L  A  V  L  A
CGATGGAACCCTTTCTGAGTTAACTCCGGTTTATTTCTATATAGCTAAAAAGGCAGATGG 1560
  D  G  T  L  S  E  L  T  P  V  Y  F  Y  I  A  K  K  A  D  G
AGGTGTGTCGACACATTTTTGTACCGATAAGCTAAGGTCATCACTAGATTATGATGGGGA 1620
  G  V  S  T  H  F  C  T  D  K  L  R  S  S  L  D  Y  D  G  E
GAGAGTGGTGTATGGGGGCACTGTTCCTGTGTTAGATGATGAAGAACTCACAATGAGGCT 1680
  R  V  V  Y  G  G  T  V  P  V  L  D  D  E  E  L  T  M  R  L
ATTGGTGGATCATTCGATAGTGGAGGGGTTTGCGCAAGGAGGAAGGACGGTTATAACATC 1740
  L  V  D  H  S  I  V  E  G  F  A  Q  G  G  R  T  V  I  T  S
AAGGGCGTATCCAACAAAAGCGATATACGAACAAGCGAAGCTGTTCTTGTTCAACAACGC 1800
  R  A  Y  P  T  K  A  I  Y  E  Q  A  K  L  F  L  F  N  N  A
CACAGGTACGAGTGTGAAAGCATCTCTCAAGATTTGGCAAATGGCTTCTGCACCAATTCA 1860
  T  G  T  S  V  K  A  S  L  K  I  W  Q  M  A  S  A  P  I  H
TCAATACCCTTTTTAATTACCGGCTATCGCTATCCTTTTTGTTATTGGTATTTATGTATC 1920
  Q  Y  P  F
TTAATTTTCTTTTAAACCTTTTTATTTGATAAATATTAGTTCTTGTTATTGTGCTTCTAG 1980
TAATAAATGAATGGTGTTATGGG                                       2003
```

A



pVS1: enh.35S-AlMV-*sst-nos*

B



pVF1: enh.35S-AlMV-*fft-nos*

Fig. 5

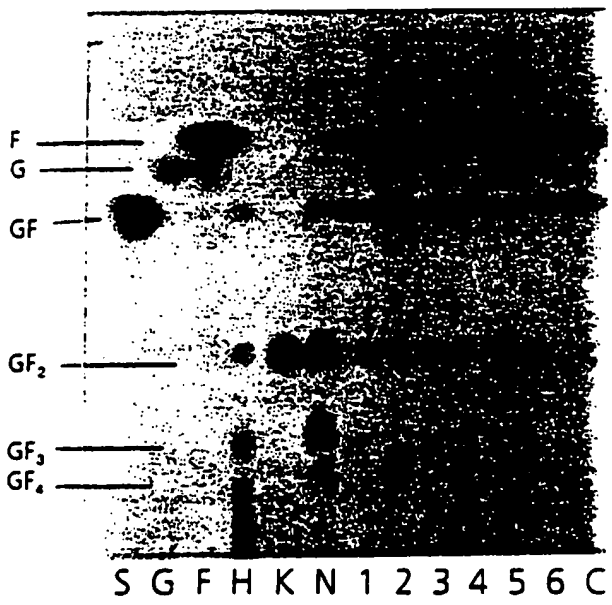


Fig. 6

Fig. 7

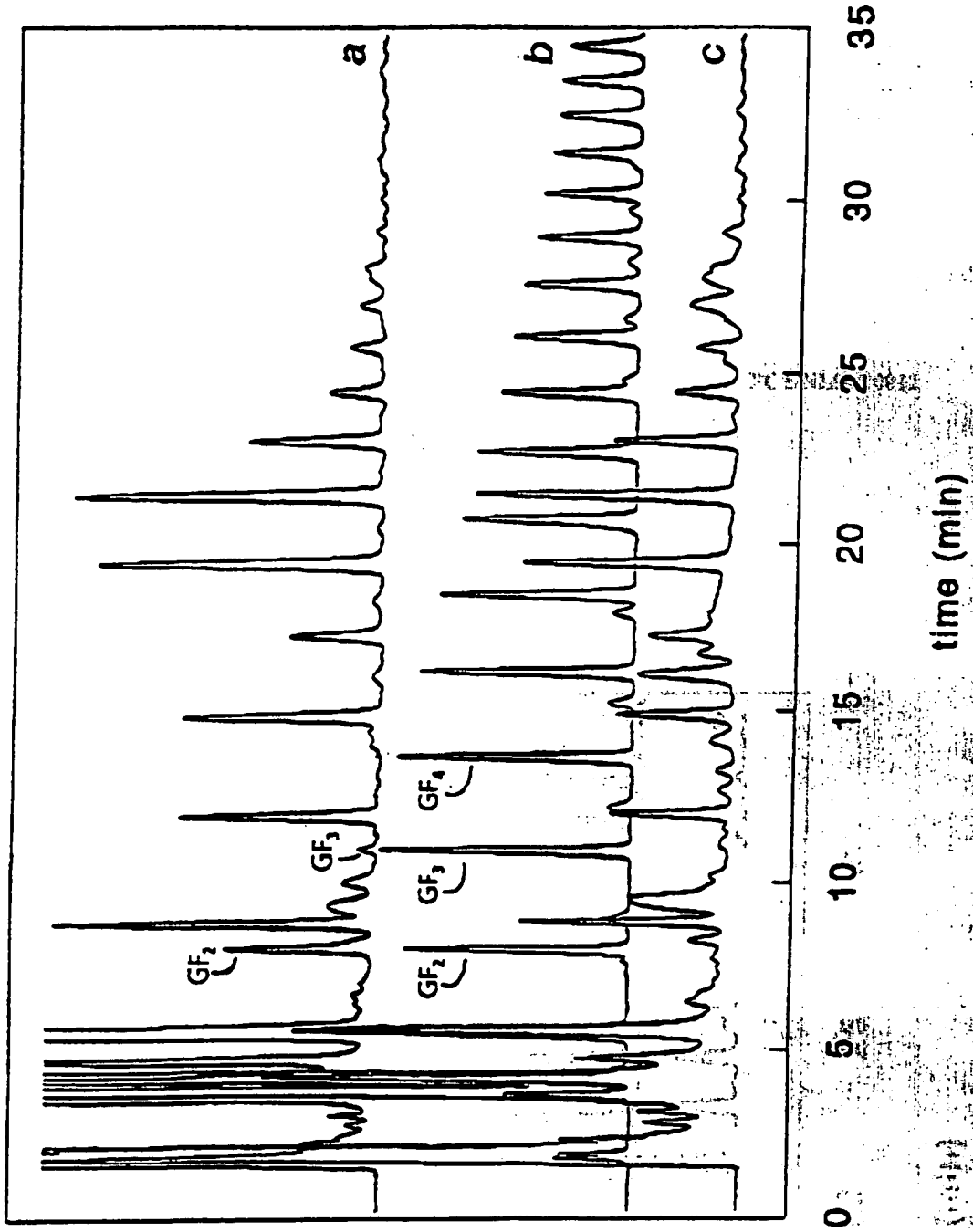
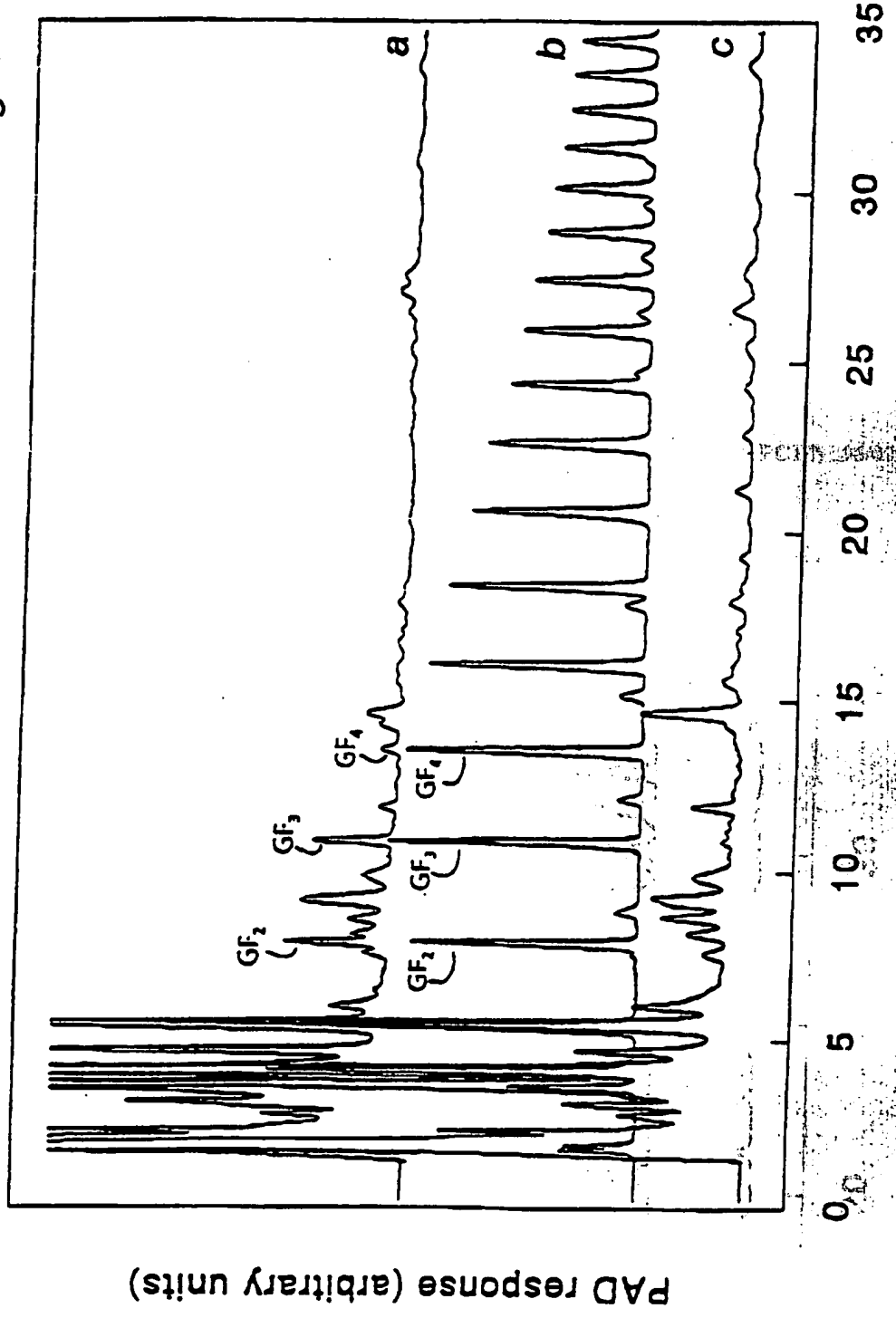

a
b
c
GF2
GF5
GF3
GF4
GF2
0
5
10
15
20
25
30
35
time (min)
PAD response (arbitrary units)

Fig. 8


a
b
c
GF2
GF3
GF4
GF2
GF3
GF4
0
5
10
15
20
25
30
35
time (min)
PAD response (arbitrary units)

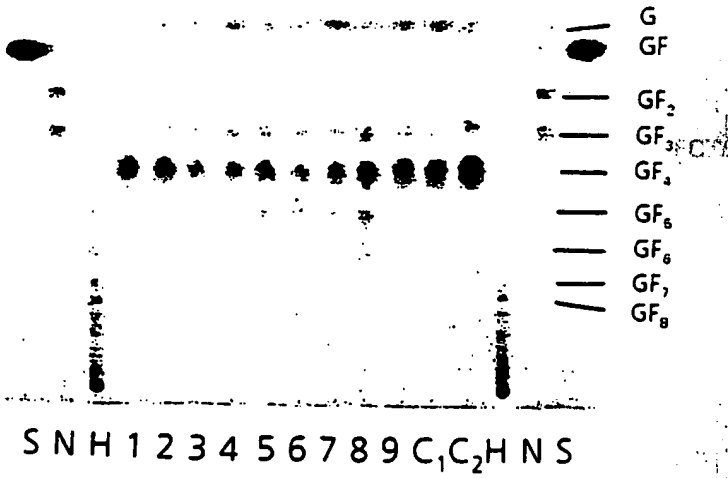


Fig. 9

INTERNATIONAL SEARCH REPORT

In ional Application No
PCT/NL 96/00012

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STUD. PLANT SCI. (1993), 3(INULIN AND INULIN-CONTAINING CROPS),173-84, XP000567837 ANGENENT, G. C., ET AL.: "Purification and properties of sucrose:sucrose fructosyltransferases from barley leaves and onion seeds" cited in the application see the whole document --- -/--	1,3-8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
22 April 1996	10-05-96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	AGRIC. BIOL. CHEM., vol. 54, 1990, pages 2429-2431, XP002001140 PRAZNIK, W., ET AL.: "Isolation and characterization of sucrose:sucrose 1F-beta-D-fructosyltransferase from tubers of Helianthus tuberosus L." cited in the application see the whole document	1,3-38
A	NEW PHYTOL., 1993, pages 717-724, XP002001141 LÜSCHER, M., ET AL.: "Purification and characterization of fructan:fructan fructosyltransferase from Jerusalem artichoke (Helianthus tuberosus L.)" cited in the application see the whole document	2-8
A	J. EXPERIMENTAL BOTANY, vol. 45, no. 280, November 1994, pages 1623-1631, XP000568904 KOOPS, A.J., ET AL.: "Purification and characterization of the enzymes of fructan biosynthesis in tubers of Helianthus tuberosus 'Colombia'. I. Fructan:fructan fructosyl transferase" cited in the application see the whole document	2-8
A	WO,A,94 14970 (STICHTING SCHEIKUNDIG ONDERZOE ;SMEEKENS JOSEPHUS CHRISTIANUS (NL)) 7 July 1994 cited in the application see the whole document	1-8
A	WO,A,89 12386 (CALGENE INC) 28 December 1989 cited in the application see page 9, line 23 - line 35	1-8
E	WO,A,96 01904 (STICHTING SCHEIKUNDIG ONDERZOE ;SMEEKENS JOSEPHUS CHRISTIANUS (NL)) 25 January 1996 see claims 6-8	1-8 2-8

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

I tional Application No
PCT/NL 96/00012

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9414970	07-07-94	AU-B-	5843794	19-07-94
		HU-A-	71782	28-02-96
		NL-A-	9300646	18-07-94
		PL-A-	309606	30-10-95
		EP-A-	0677112	18-10-95
WO-A-8912386	28-12-89	AU-B-	3852089	12-01-90
WO-A-9601904	25-01-96	NL-A-	9401140	01-02-96
		NL-C-	1000064	08-01-96
		AU-B-	2809195	09-02-96